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THE UPTAKE OF PROTEINS BY THE CHLOROPLAST AND
THEIR SUBSEQUENT ROUTING TO THE THYLAKOID
TITLE

AUTHOR Jamie B. Shackleton

DEGREE

AWARDING BODY University of Warwick

DATE September 1991

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**THE UPTAKE OF PROTEINS BY THE CHLOROPLAST AND
THEIR SUBSEQUENT ROUTING TO THE THYLAKOID**

By

Jamie B. Shackleton

**Submitted to the University of Warwick
for fulfilment of the requirement for the
Degree of Doctor of Philosophy**

**Biological Sciences Department
University of Warwick
Coventry**



September 1991

Summary

Proteins located in the chloroplast are encoded by both the chloroplast and the nuclear genomes. Those encoded by the nuclear genome are translated on cytosolic ribosomes as precursors with N-terminal pre-sequences. These pre-sequences target the proteins to their correct location within the chloroplast. Proteins destined for the thylakoid lumen contain a bipartite pre-sequence and are imported by a presumed two step process. The envelope transfer domain (ETD) targets the protein across the chloroplast envelope into the stroma where the precursor is cleaved to an intermediate by the stromal processing peptidase (SPP). This stromal intermediate is targeted by the thylakoid transfer domain (TTD) across the thylakoid membrane into the lumen where it is cleaved to the mature size by the thylakoidal processing peptidase (TPP).

A cDNA clone encoding the precursor of the 33kDa protein (pre-33K) of the photosynthesis oxygen evolving complex (OEC), a nuclear encoded thylakoid lumen protein, was isolated and the section encoding the N-terminal pre-sequence was sequenced. This cDNA clone was used to confirm the two step model proposed for the targeting of thylakoid proteins. The question of whether import into the thylakoid lumen occurs via a stromal intermediate has been addressed by attempting to block the SPP recognition site using iodoacetate (IAA). By blocking the recognition of the SPP cleavage site of the precursor of the 23kDa protein (pre-23K) of the OEC, it was shown that the import of precursor protein into isolated chloroplasts and thylakoids can occur without the generation of the stromal intermediate. Import of this blocked precursor into the thylakoid lumen is assumed to occur by a one step process and represents the first demonstration of one step import. The TTD in this one step import model acts as an internal targeting signal, clearly indicating that the thylakoid targeting signal does require an N-terminal position to target a protein across the thylakoid membrane. When the pre-33K was treated with iodoacetate a stromal intermediate was generated but this failed to target across the thylakoid membrane (possibly due to a failure of the TTD to recognise the thylakoid receptor).

Sequence analysis of thylakoid transfer domains has revealed a Ala X Ala conserved motif immediately before the TPP cleavage site which appears to be required for TPP cleavage. Site directed mutagenesis of the 33K precursor was used to define the reaction specificity of the TPP. A number of mutant proteins have been identified which are blocked for TPP cleavage when processed in vitro. These mutant proteins when imported into isolated chloroplasts are located in the thylakoid lumen and either processed to a 36kDa intermediate or to 33K and the 36kDa intermediate. These mutant proteins were expressed in Escherichia coli and were exported to the periplasmic space where they were either processed to 33K or to an intermediate slightly larger than 33K. When the reaction specificities of bacterial leader peptidase, eukaryotic signal peptidase and TPP (Fikes et al., 1990 and Folz et al., 1988 have carried out a similar site directed mutagenesis study to that carried out for TPP above) were compared, it was found that although all three peptidases possessed similar reaction specificities they were not identical.

ACKNOWLEDGEMENT

I would like to thank the following for their assistance during the course of this project: Dr. Colin Robinson, for his assistance and constructive criticism throughout the course of the work; the members of the Plant Biochemistry Group, in particular those of the C.R. group; Kate Day and all my friends for their support; the trustees of the Magnus Educational Foundation and Mrs. C. Thomas for their generous support.

DECLARATION

The work contained in this thesis was the result of original research conducted by myself under the supervision of Dr. C. Robinson. All sources of information have been acknowledged by means of reference. None of the work has been used in any previous application for a degree.

The cloning of the cDNA encoding pre-33K was done in collaboration with Dr. J.W. Meadows (Warwick). I would like to thank H.James (Warwick) for providing the cDNA clone for pre-23K and figure 13. Dr. C. Howe (Cambridge) kindly carried out the prediction of signal peptidase cleavage sites within the 33K pre-sequence (figure 36). Antisera to pea 33K was prepared by J. Musgrove (Warwick). Microsequencing of cleaved proteins was carried out in collaboration with B.Dunbar (Aberdeen). D. Bassham (Warwick) provided the stromal intermediate microsequence data and Dr. P. Elderfield (Warwick) provided the mature protein microsequence data (figures 15 and 16).

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Table 1 Summary of reaction specificities of TPP,
LEP and SP based on the data presented
above and in Fikes et al., (1990) and
Folz et al., (1988)

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LIST OF ABBREVIATIONS

Ala	alanine
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
Bis	N'N'-methylene-bisacrylamide
CAT	chloramphenicol acetyltransferase
cAMP	adenosine 3',5'-cyclic monophosphate
cDNA	complementary DNA
cpDNA	chloroplast DNA
Ci	Curie (3.7×10^{10} disintegrations per second)
CIP	calf intestinal alkaline phosphatase
C-terminal	carboxyl terminal
cytb ₂	cytochrome b ₂
cyt b ₆	cytochrome b ₆
cyto f	cytochrome f
dATP	2'deoxyadenosine 5'triphosphate
dCTP	2'deoxycytidine 5'triphosphate
ddATP	2',3'dideoxyadenosine 5'triphosphate
ddCTP	2',3'dideoxycytidine 5'triphosphate
ddGTP	2',3'dideoxyguanosine 5'triphosphate
ddTTP	2',3'dideoxythymidine 5'triphosphate
DTT	dithiothreitol

DGDG	digalactosyldiacylglycerol
dGTP	2'deoxyguanosine 5'triphosphate
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
dNTPs	deoxyribonucleoside triphosphates
DP	docking protein
dTTP	2'deoxythymidine 5'triphosphate
<u>E.coli</u>	<u>Escherichia coli</u>
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethyleneglycol-bis (-amino ethyl ether) N,N'-tetra acetic acid
E.R.	endoplasmic reticulum
ETD	envelope transfer domain
Fd	ferredoxin
GIP	general insertion protein
GTP	guanosine triphosphate
H ₂ O	sterile water
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid
HPLC	high pressure liquid chromatography
IAA	iodoacetate
IMPI	inner membrane protease I
int-	intermediate
kbp	kilo base pair
kDa	kilodalton
<u>lac Z</u>	beta-galactosidase
LEP	leader peptidase
Leu	leucine

LHCI	light harvesting complex I
LHCII	light harvesting complex II
LHCP	light harvesting chlorophyll protein
LMP	low melting point
LSP	prolipoprotein signal peptidase
Lys	lysine
M	molar
MGDG	monogalactosyldiacylglycerol
MBP	maltose binding protein
MOM	mitochondrial outer membrane protein
MPP	matrix processing peptidase
mRNA	messenger RNA
NADP	nicotinamide adenine dinucleotide phosphate
N-terminal	Amino terminal
OD	optical density
OEC	oxygen evolving complex
PC	plastocyanin
PEG	polyethylene glycol
PEP	protease enhancing protein
pfu	plaque forming unit
PG	phosphatidylglycerol
pheo	pheophytin
PMF	proton motive force
PMSF	phenylmethylsulphonyl fluoride
prC5	pre-sequence of cytochrome oxidase subunit Va
pre-	precursor
PSI	photosystem I
PSII	photosystem II
RF	replicative form

RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
r.t.	room temperature
Rubisco	ribulose-1,5-bisphosphate carboxylase
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SIM	sucrose isolation medium
SOD	superoxide dismutase
SP	signal peptidase
SPP	stromal processing peptidase
SRP	signal recognition particle
ss	single stranded
SSR	signal sequence receptor
SSU	small subunit
TBE	tris-borate-EDTA
TEMED	N,N,N,N,'-tetramethyl diamine
TF	trigger factor
TPP	thylakoid processing peptidase
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
tRNA	transfer RNA
TTD	thylakoid transfer domain
u.v.	ultra violet
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl beta-D-galactoside
³⁵ [S] met	³⁵ sulphur labelled methionine
³ [H] lys	tritium labelled lysine
³ [H] leu	tritium labelled leucine

23K mature 23kDa OEC polypeptide
33K mature 33kDa OEC polypeptide

SINGLE AND TRIPLE LETTER AMINO ACID CODE

A - Ala	alanine
R - Agr	arginine
N - Asn	asparagine
D - Asp	aspartic acid
C - Cys	cysteine
Q - Gln	glutamine
E - Glu	glutamic acid
G - Gly	glycine
H - His	histidine
L - Leu	leucine
I - Ile	isoleucine
K - Lys	lysine
M - Met	methionine
F - Phe	phenylalanine
P - Pro	proline
S - Ser	serine
T - Thr	threonine
W - Trp	tryptophan
Y - Tyr	tyrosine
V - Val	valine

SECTION I - LITERATURE REVIEW

Section 1:1 INTRODUCTION

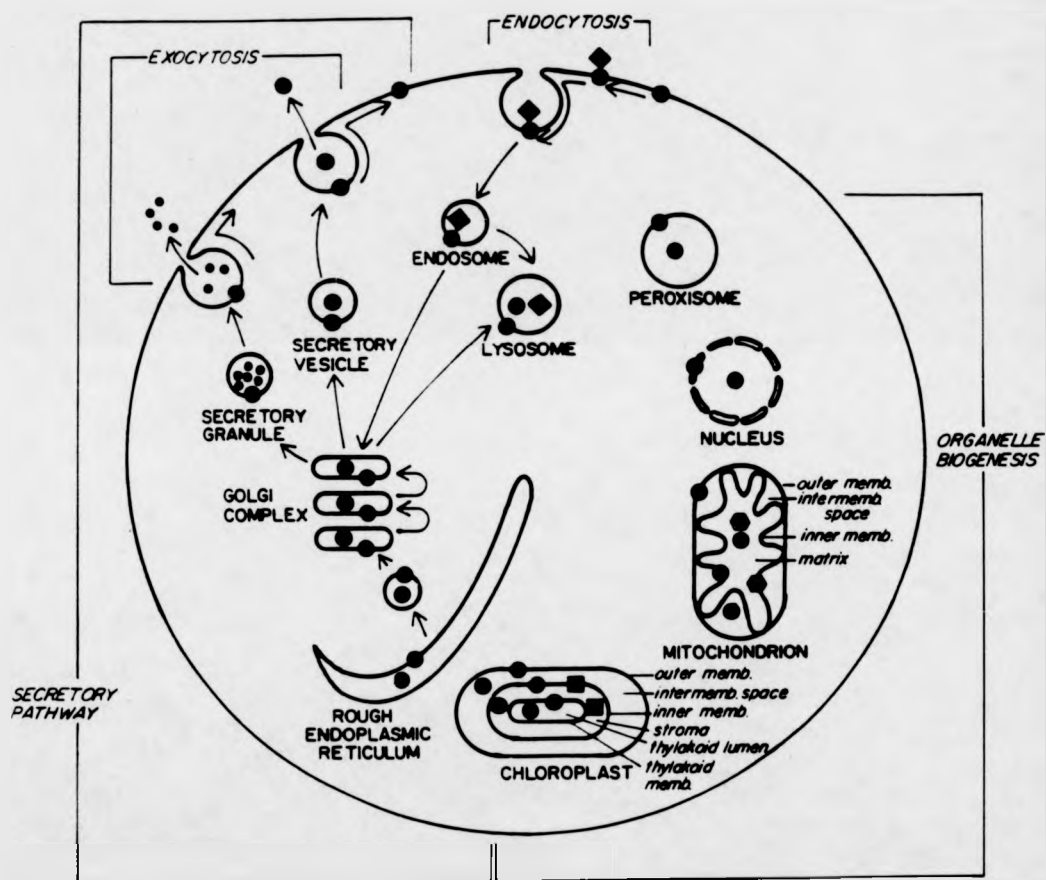
The localization of proteins to specific membrane-bound compartments allows the segregation of the various biochemical functions which characterise the eukaryotic cell. Without the compartmentalization made possible by the lipid bilayer, many of the biochemical processes essential to the eukaryotic organism could not take place. For example, the chloroplast provides the correct environment for photosynthesis to take place, the mitochondria for oxidative phosphorylation and the lysosome for degradation of endocytosed proteins. Nearly all the proteins synthesised by an eukaryotic cell are nuclear encoded, although a small number are encoded by the chloroplast and mitochondrial genomes. These nuclear encoded proteins are synthesised in the cytosol of the cell and those that do not function in the cytosol are targeted to their respective compartment; figure 1 illustrates the complexity of protein targeting in eukaryotic cells. As can be seen from figure 1 proteins are targeted to a wide range of compartments within the eukaryotic cell; the work I have undertaken concerns the targeting of nuclear encoded proteins to the thylakoid lumen of the chloroplast.

Section 1:2 The Structure And Function Of Chloroplasts

The chloroplast is a member of the plastid family and is characterised by the presence of the green pigment chlorophyll

Figure 1 Protein targeting within the eukaryotic cell

Simplified representation of an eukaryotic cell showing the sites to which targeted proteins are directed. Nuclear-encoded proteins (●) are directed to a variety of sites. Rough endoplasmic reticulum-dependent protein traffic (the secretory pathway) represents the major protein route by which nuclear-encoded proteins are directed into and through the endoplasmic reticulum to lysosomes and via secretory vesicles (constitutive secretion) or secretory granules (regulated secretion) to the plasma cell membrane or to the outside of the cell. Four types of organelles are shown. Chloroplasts and mitochondria have small genomes coding for a limited number of proteins localized to the chloroplast stroma and inner membrane (■) or to the mitochondrial matrix and inner membrane (◆). Other chloroplast and mitochondrial proteins, and all nuclear and peroxisomal proteins are encoded by the nucleus and are imported from the cytoplasm. Proteins synthesised by other cells (◆) are imported via endocytosis, as are plasma membrane proteins. Some of these proteins may be redirected to the cell surface, or they may be routed into lysosomes or other intracellular targets. (Taken from "Protein targeting", by A.P.Pugsley, 1989)



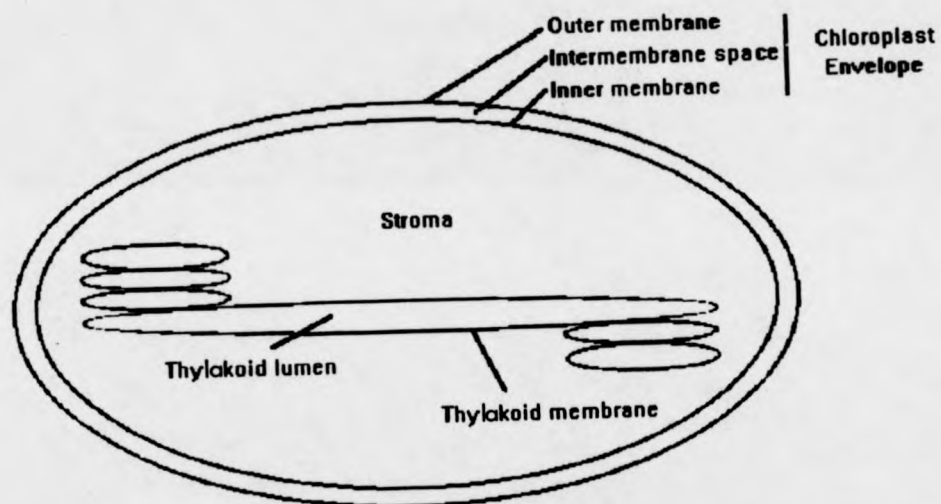
and by being the site of the photosynthetic reaction (Schnepf, 1980). Chloroplasts are also the site of nitrite and sulphate reduction and their assimilation into amino acids, as well as being a major site for lipid synthesis (Douce and Joyard, 1990). The chloroplast can be regarded as consisting of three major regions: (i.) a double membrane envelope consisting of an outer and inner membranes with an intermembrane space between; (ii.) a soluble phase known as the stroma; (iii.) a membranous network within the stroma known as the thylakoids, consisting of a thylakoid membrane and a thylakoid lumen (the lumen being a continuous soluble phase). These structural features are summarised in figure 2.

The chloroplast envelope has a role in the import of cytosolic molecules and proteins as well as being a major site of lipid synthesis. The outer membrane of the chloroplast envelope is permeable to molecules up to 10kDa whereas the inner membrane is relatively impermeable and contains a number of specific translocators (Flugge and Benz, 1984; Douce and Joyard, 1990). The stroma contains the chloroplast DNA (cpDNA) and the protein synthesising machinery as well as the enzymes required for the reduction of carbon dioxide and its assimilation into carbohydrates, amino acids, fatty acids and terpenoid compounds (Boulter et al., 1972; Douce and Joyard, 1990). The thylakoids consist of a network of membranes surrounding an intra thylakoid space known as the thylakoid lumen (Whitmarsh, 1986). The thylakoid membrane has a role in protein targeting, as well as containing the protein complexes required for energy transduction in the chloroplast.

The chloroplast contains three sets of membranes: the outer

Figure 2 The six phases of the chloroplast

A schematic representation of the chloroplast showing the outer and inner chloroplast envelope with the intermembrane space between, the soluble stromal phase containing the thylakoids which are composed of a soluble thylakoid lumen surrounded by the thylakoid membrane.



chloroplast envelope, inner chloroplast envelope and the thylakoid membrane. These membranes contain different sets of proteins and the lipid content of these membranes is also different (Douce and Joyard, 1990). All three plastid membranes are characterised by a low phospholipid content and by the presence of large amounts of galactolipids such as monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). The outer envelope membrane is enriched in DGDG, whereas the inner envelope membrane is enriched in MGDG. The major phospholipid of the outer envelope membrane is phosphatidylcholine whereas phosphatidylglycerol (PG) is the major phospholipid of the inner envelope membrane and the thylakoid membrane; the inner envelope membrane and the thylakoid membrane contain no phosphatidylcholine.

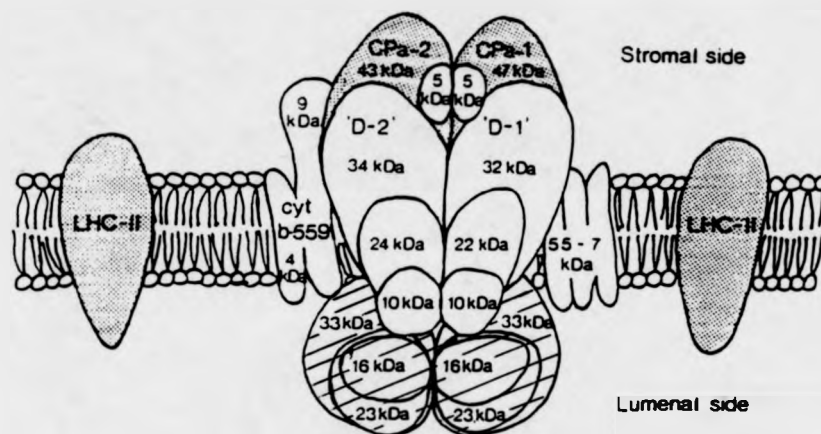
Section 1:3 The Function Of The Oxygen Evolving Complex
 Polypeptides

The polypeptides used in this study of the mechanisms of protein import into chloroplasts and intra-organellar targeting to the thylakoid lumen were the precursors of the 33kDa and 23kDa proteins (33K and 23K) of the oxygen evolving complex (OEC). These two polypeptides, together with the 16kDa OEC polypeptide are located on the luminal side of the thylakoid membrane as part of photosystem II (PSII) (figure 3) and function to stabilize the Mn involved in the splitting of water during photosynthesis.

PSII forms one part of the light harvesting system required for the reduction of NADP^+ by electrons generated from the proteolysis of water, as outlined in the Z-scheme figure 4. PSII

Figure 3 Schematic illustration of the composition and organization of the photosystem II complex of plant thylakoids

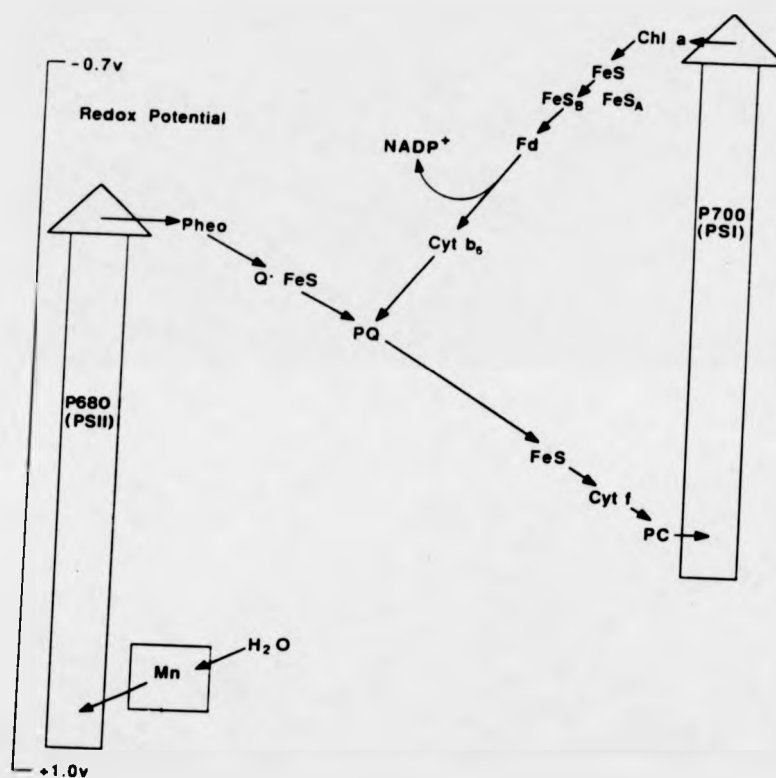
The polypeptide composition of photosystem II is shown. The photosystem is composed of a core complex, a light harvesting complex (LHC) and an oxygen evolving complex (OEC). The OEC is located on the luminal side of the thylakoid membrane and is composed of the 33kDa, 23kDa and 16kDa polypeptides. (Adapted from "Transbilayer organization of photosystem II proteins with special emphasis on the 32kDa Q_b -binding protein, by Andersson et al., 1987)



OEC polypeptides

Figure 4 The Z-scheme for the reduction of NADP^+ by photosynthesis

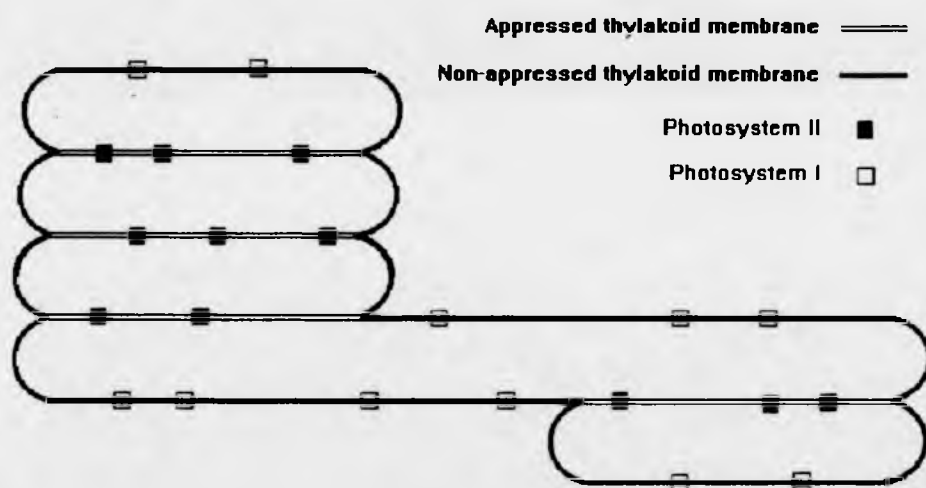
The cooperation of two light reactions in the reduction of NADP^+ from electrons derived from water is shown. The redox scale gives an indication of the redox potentials developed by the photosystems. Abbreviations used are: PSII, photosystem II; pheo, pheophytin; Q.FeS, bound plastocyanin and an iron-sulphur centre; cyt f, cytochrome f; PC, plastocyanin; PSI, photosystem I; Fd, ferredoxin; cyt b_6 , cytochrome b_6 . (Adapted from "The biochemistry of energy utilization in plants", by D.T.Dennis, 1987).



is composed of a core complex, a light-harvesting complex (LHCII) and an extrinsic complex including the OEC polypeptides. A photon of light energy is absorbed by the LHCII and is passed onto chlorophyll a in the core complex, where an electron is raised into a new orbital. This electron is then passed onto a number of acceptor molecules and then to plastoquinone. The result of the removal of the electron from chlorophyll a is to leave chlorophyll a with a net positive charge. This positive charge is neutralised by electrons resulting from the splitting of water. The generation of one molecule of oxygen from the splitting of water results in the generation of four electrons. PSII must therefore have a mechanism for the storage of oxidative equivalents until four are available for water to be split with the release of molecular oxygen. The most likely candidate for an oxidative store is the Mn stabilized by the OEC polypeptides. The other major component of the light harvesting system is photosystem I (PSI). Again a photon of light energy collected by LHCI is passed onto chlorophyll a and results in the raising of an electron to a new potential. This electron is passed via a number of acceptors to finally reduce NADP^+ to NADPH. The now positively charged PSI is neutralized by electrons from plastoquinone via a number of acceptors including plastocyanin. The order of events outlined above is summarised in the Z-scheme in figure 4. The two photosystems discussed above, although linked biochemically, have separate physical locations in the thylakoid membrane, PSI being found in non-appressed membranes and PSII being found mainly in the appressed membranes (figure 5).

Figure 5 The distribution of photosystems I and II within the thylakoid membrane

The appressed and non-appressed thylakoid membranes are schematically represented and the distribution of photosystems I and II within the thylakoid membrane is indicated.



Section 1:4 Protein Synthesis In Plant Cells

Plant cell proteins are encoded by three distinct genomes: nuclear, chloroplast and mitochondrial. Most higher plant cp DNAs are known to contain only chloroplast rRNA genes, tRNA genes and approximately 100 genes coding for protein synthesised in the chloroplast (Ellis, 1984). All other proteins involved in chloroplast biogenesis are nuclear encoded. These nuclear encoded proteins are synthesised as precursors in the cytoplasm of the plant cell, after which they are targeted post-translationally into the chloroplast (Ellis, 1984; Ellis & Robinson, 1987). The chloroplast has six distinct phases: the outer chloroplast envelope membrane, inner chloroplast envelope membrane, intermembrane space, stroma, thylakoid membrane and thylakoid lumen, as shown schematically in figure 2. Nuclear encoded proteins are targeted to all six phases. Of special interest are those nuclear encoded proteins that are targeted to the thylakoid lumen, such as 33K and 23K, as they have to traverse the five other phases in order to reach their final destination.

Much useful information about the mechanism of protein targeting into the chloroplast has come from studies on the sequence analysis of precursors, the energetics and processing events of targeting, and the retargeting of proteins. A comparison of chloroplast protein targeting with that in other systems may also be informative about possible shared mechanisms of targeting. These areas are considered below.

Section 1:5 Two Step Model For Targeting To The Thylakoid Lumen

Chloroplast proteins which are nuclear encoded are synthesised in the cytosol of the plant cell as precursors with N-terminal pre-sequences. These pre-sequences are required for targeting into the chloroplast. A model for the targeting of nuclear encoded thylakoid lumen located precursors has arisen from the sequence analysis, processing studies, and from studies on the targeting of foreign proteins (as discussed below). This model regards import across the chloroplast envelope and the thylakoid membrane as two separate events.

The precursor is first synthesised in the cytosol of the plant cell as a precursor consisting of two domains; an N-terminal envelope transfer domain (ETD) followed by a thylakoid transfer domain (TTD). After synthesis the ETD binds to the chloroplast envelope, probably via a protein component or receptor (section 1:9:a). Transport of the precursor across the chloroplast envelope is then mediated by the ETD; the mechanism of translocation is unknown, but ATP is required (section 1:10:a). The ETD is proteolytically removed by stromal processing peptidase (SPP) in the stromal compartment to generate a stromal intermediate, consisting of the TTD and the mature protein. The TTD then associates with the thylakoid membrane, possibly via an electrostatic interaction between the +ve N-terminal end of the TTD and the overall -ve charged thylakoid membrane. A protein component / receptor would also appear to be involved at this or a later stage (section 1:9:b). The TTD then mediates translocation across the thylakoid membrane by a mechanism which is poorly understood, although a pH gradient is known to be required (section 1:10:b). Either during or shortly after import across the thylakoid membrane the TTD is proteolytically removed

by thylakoid processing peptidase (TPP) to generate a mature-size product. The events of the two step import model are summarised in figure 6. After the generation of the mature protein there is a third step for some proteins (such as those of the OEC); that of association with one of the thylakoid membrane complexes. Very little is known about the mechanisms of assembly.

The evidence supporting the two step model is discussed in the following sections. The targeting of proteins to chloroplast compartments other than the thylakoid lumen is also considered with special emphasis given to the large body of work on the targeting of nuclear encoded proteins to the stroma. Implicit in this discussion is the assumption that targeting across the chloroplast envelope of stromal and thylakoid lumen proteins occurs via a common import mechanism; it should, however, be noted that there is little proof of this. Buvinger *et al.*, (1989) showed that import of the small subunit of Rubisco (stroma targeted), light-harvesting chlorophyll a/b protein II (LHC II)(thylakoid membrane targeted) and other unidentified polypeptides could be inhibited by a synthetic peptide to the first 20 residues of the LHC II pre-sequence; suggesting a common import pathway.

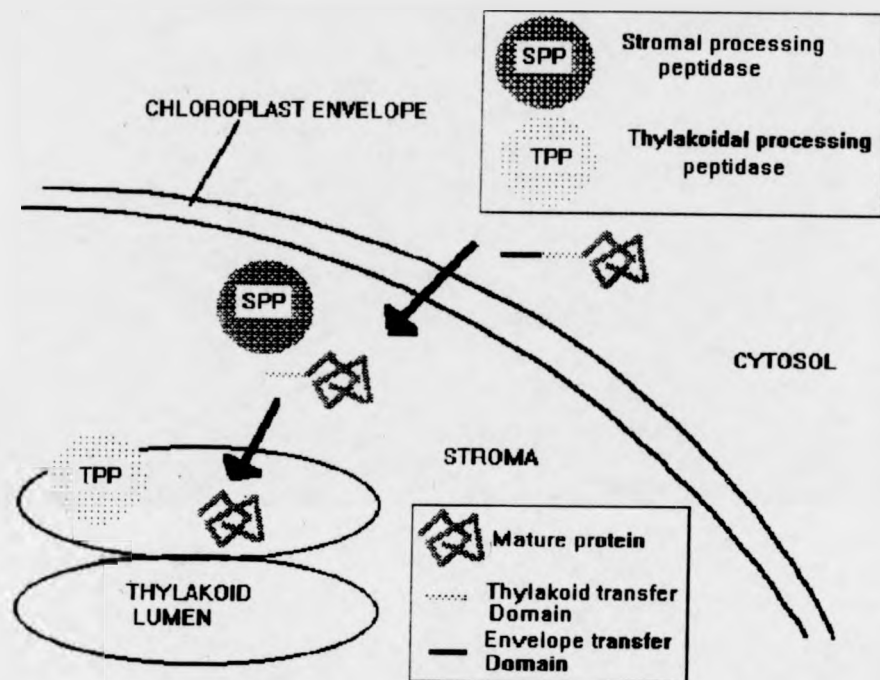
Section 1:6 Sequence Analysis Of Targeting Signals

Section 1:6:a Analysis Of Pre-sequences Used To Target Proteins Into Chloroplasts

Nuclear-encoded chloroplast proteins are synthesised in the cytosol of the plant cell as precursors with N-terminal pre-sequences. A number of chloroplast precursors have been cloned

Figure 6 A schematic representation of protein transport into chloroplasts and intra organellar routing to the thylakoid lumen

The protein is synthesised in the cytoplasm as a precursor with an N-terminal targeting signal. The precursor is then imported across the chloroplast envelope into the stroma where the N-terminal section of the targeting signal (the envelope transfer domain) is removed by the stromal processing peptidase (SPP). The stromal intermediate is then targeted across the thylakoid membrane where the remaining section of the targeting signal (the thylakoid transfer domain) is removed by the thylakoidal processing peptidase.



and sequenced, most of them have N-terminal pre-sequences. These N-terminal pre-sequences, which are removed during or shortly after import, fall into two classes: those that are targeted across the chloroplast envelope and those that are targeted across both the chloroplast envelope and the thylakoid membrane. In the first class fall proteins that are targeted to the stroma, such as the small subunit of ribulose 1,5-bisphosphate carboxylase (Rubisco) (Cashmore, 1983). Into the second class fall proteins targeted to the thylakoid lumen such as plastocyanin and the OEC proteins (Smeekens et al., 1985). The pre-sequences of proteins targeted to the thylakoid lumen are bipartite in nature, having a N-terminal envelope transfer domain (ETD) followed by a thylakoid transfer domain (TTD) (Hageman et al., 1986; Smeekens et al., 1986)(figure 7).

Based on the analysis of chlorophyll a/b-binding protein (LHCP) it would appear that proteins targeted to the thylakoid membrane are first targeted across the chloroplast envelope by an ETD in a manner similar to the small subunit of rubisco. LHCP then integrates into the thylakoid membrane by virtue of targeting signals located in the mature protein (Lamppa, 1988; Viitanen et al., 1988). Very few proteins targeted to the chloroplast envelope have been analysed. The nuclear encoded inner envelope targeted triose phosphate-3-phosphoglycerate phosphate translocator has been cloned and sequenced (Flugge et al., 1989); it contains an N-terminal pre-sequence which is processed when imported into isolated chloroplasts. A 37kDa polypeptide targeted to the inner membrane of the chloroplast envelope has also recently been cloned and sequenced (Flugge et al., unpublished result).

Figure 7 The primary structures of the targeting sequences of a number of chloroplast proteins

The single letter code of the targeting signals of ribulose-1,5-bisphosphate carboxylase/oxygenase SSU (Von Heijne et al., 1989) and plastocyanin of Silene (Smeekens et al., 1985). The SPP (*) and TPP (#) cleavage sites are shown, as are the envelope transfer domains (ETD) and thylakoid transfer domains (TTD). A more extensive listing and comparison has been carried out recently by Von Heijne et al., (1989).

Stroma: Ribulose-1,5-bisphosphate carboxylase/oxygenase SSU of spinach (Von Heijne et al., 1989)

ETD MASSVLSAAVATVSRTPAQASMWAPFTGLKSTVGFPATKNDITSLASNGGRVQC*

Thylakoid lumen: Plastocyanin of Silene (Smeekens et al., 1985)

ETD MATVTSSAAVAIPSFAGLKASSTTRAATVSTISRSKVAVATPRMSIK*

TTD ASLKDVGVVVAATAAGILAGNAMA#

The pre-sequences of these two precursors show no similarity with the ETDs of other chloroplastic precursors, but they do show amphiphilic structures characteristic of mitochondrial targeting signals (Flugge, 1990). The 6.7kDa polypeptide identified by Salomon et al. (1990) remains the only outer membrane protein of the chloroplast envelope to have been cloned and sequenced; it does not contain a cleavable N-terminal targeting signal, and presumably intergrates into the outer membrane of the chloroplast envelope by virtue of an internal targeting signal.

Figure 8 compares the pre-sequences of the nuclear encoded thylakoid lumen targeted pre-33K of the cyanobacterium Anacystis nidulans with that of the higher plant spinach (Kuwabara et al., 1987; Tyagi et al., 1987). The higher plant pre-sequence is bipartite, consisting of an ETD followed by a TTD, whereas that of the cyanobacterium only consists of a TTD. The cyanobacterial pre-33K only has to cross the thylakoid membrane whereas the higher plant protein has to cross the chloroplast envelope and the thylakoid membrane. The endosymbiont hypothesis as put forward by Schwartz & Dayhoff, (1978) envisages a cyanobacterial progenitor of the chloroplast which lived in a stable endosymbiotic relationship with a eukaryotic cell. Eventually some of the genes of the chloroplast progenitor were transferred to the host plant cell. The proteins of these transferred genes which still had to function in the chloroplast progenitor would have to be targeted back to the chloroplast. This was achieved by the addition of an ETD. Those cyanobacterial genes which encoded proteins located in the thylakoid lumen on transfer to the

Figure 8 The protein sequences of pre-33K proteins of
spinach and Anacystis nidulans

The single letter code of the 33kDa proteins of spinach and A.nidulans are shown. The putative SPP (*) and TPP (#) cleavage sites are shown.

Pre-33K of spinach (Tyagi et al., 1987)

MAASLQASTFELQPTKVASRNTLQLRSTQNVCK*AFGVESASSGGRSLSLQSDLKELANKCVDATKLAGLALATSALLIASGANA#

Pre-33K of Anacytis nidulans R2 (Kuwabara et al., 1987)

MRVRAFLAFLAVCLGVLTACSSGPTAA#

nuclear genome, would be targeted back to the stromal compartment by virtue of the addition of an ETD and then the ancestral TTD would target them into the thylakoid lumen (Ellis, 1984).

Section 1:6:b Sequence Analysis Of Envelope Transfer Domains

Analysis of a number of ETDs has revealed some common features (von Heijne et al., 1989; Keegstra & Olsen, 1989). At the primary sequence level the only conserved region appears to be a N-terminal methionine-alanine sequence. The ETD has an overall +ve charge and it tends to contain a high proportion of amino acids containing aliphatic hydroxyl side chains (serine and threonine). There is almost a complete absence of aspartate, glutamate and tyrosine residues in the ETD. At the secondary structure level there is a conserved amphiphilic beta-strand immediately before the stromal cleavage site. Within this region there is also a high proportion of arginine residues and a low proportion of leucine residues. Turn inducing residues such as glycine, serine, aspartate, asparagine or proline are absent from the region immediately around the stromal cleavage site. The roles of these primary and secondary structures in targeting across the chloroplast envelope and proteolytic cleavage by SPP are unclear.

Section 1:6:c Sequence Analysis Of Thylakoid Transfer Domains

The analysis of TTDs has also revealed a number of common features (von Heijne et al., 1989; Keegstra & Olsen et al., 1989). At the primary sequence level there tends to be small

hydrophobic residues located at the -3 and -1 residues (+1 would be the first residue of the mature protein). A 5-6 residue region immediately before the TPP cleavage site tends to contain small hydrophobic residues such as alanine and is usually preceded by a turn-inducing residue such as glycine, serine, aspartate, asparagine or proline. The central region of the TTD contains a 7-15 residue stretch of hydrophobic amino acids. The N-terminal region of the TTD has a small net positive charge.

Bacterial export signals share a number of common features with the thylakoid import signals. An N-terminal +ve charge, a central hydrophobic domain and small hydrophobic residues at the -3 and -1 positions are seen in both TTDs and bacterial export signals (von Heijne, 1985; von Heijne *et al.*, 1989). These conserved features have led to the suggestion that the thylakoid import and bacterial export mechanisms may be similar (sections 1:11:b:iii and 1:12:a).

The targeting of nuclear encoded proteins to the thylakoid lumen is also similar in some respects to the targeting of mitochondrial intermembrane space proteins. They both have bipartite pre-sequences, the first domain targets the chloroplast or mitochondrial precursor across their respective organellar envelopes. The second domain, in the case of the chloroplast targeted precursor, targets the intermediate into the thylakoid lumen, while the second domain in the mitochondrial precursor targets the intermediate into the mitochondrial intermembrane space. The TTD and mitochondrial inner envelope targeting signals share a number of other common features. They both have an overall +ve charge at their N-terminus, a central hydrophobic domain and have small hydrophobic residues located before their

final cleavage site (von Heijne et al., 1989). A crude extract of the protease responsible for the cleavage of polypeptides targeted to the mitochondrial intermembrane space has been isolated (Schneider et al., 1991); it remains to be shown if this protease has a similar reaction mechanism to TPP (also see section 1:11:a:ii). It should be noted that the targeting signal for the inner chloroplast envelope membrane (discussed earlier) does not share any features with that for the inner mitochondrial envelope membrane.

Section 1:6:d Chlamydomonas reinhardtii Targeting Signals And Dual Targeting

Von Heijne et al., (1989) showed that higher plant ETDs and mitochondrial targeting signals share no structural similarities except for being rich in basic and hydroxylated residues, containing few acidic residues and lacking extensive hydrophobic regions. However, in the comparison by Franzen et al., (1990) of the ETDs of C.reinhardtii and mitochondrial targeting signals, a number of common features were observed, including the ability to form amphipathic structures thought to be a key requirement for mitochondrial targeting. These similarities may explain the earlier observation of Hurt et al. (1986) that the C.reinhardtii rubisco SSU pre-sequence could target DHFR into yeast mitochondria, suggesting that the C.reinhardtii pre-sequence may be recognised by the mitochondrial import machinery. Lacoste-Royal and Gibbs, (1985) also showed by in-situ hybridization with antibodies to the rubisco SSU that the mitochondria of the chrysophyte alga Ochromonas doncia contained mis-targeted

rubisco SSU. From the above observations it is likely that some algal chloroplast pre-sequences may be recognised by the mitochondria import machinery.

Huang et al., (1990) used the pre-sequence of the mitochondrion-targeted cytochrome oxidase subunit Va (prC5) to target the reporter protein chloramphenicol acetyltransferase (CAT) into both mitochondria and chloroplasts. The prC5/CAT chimeric protein was taken up by both organelles with similar efficiencies and processed to the same sized mature protein. The prC5 has a number of unique features for a mitochondrial pre-sequence (i. it forms an amphiphilic antiparallel beta-sheet instead of the usual amphiphilic alpha-helix; ii. it imports efficiently at 0°C into mitochondria; and iii. it shows import into protease treated mitochondria) which may explain this dual targeting. Huang et al., (1990) suggest that dual targeting into mitochondria and chloroplasts may occur with a small subset of polypeptides but this does not represent the rule.

The specificity of protein targeting into the chloroplast has been demonstrated using immunogold labelling by de Boer et al., (1988) who demonstrated that antibodies to plastocyanin (a thylakoid lumen protein) only labelled the thylakoid lumen and were not observed in the chloroplast stroma, other organelles or the cytoplasm. Whelan et al., (1990) have shown that the mitochondrion F₁-ATPase beta-subunit was only targeted to mitochondria and not to chloroplast. In the same study it was shown that the chloroplast targeted pre-33K was not taken up by isolated plant mitochondria, but pre-33K was not taken up efficiently by isolated chloroplast either. Boutry et al., (1987) have shown that a chimeric protein containing the Nicotiana

plumbaginifolia F₁ beta ATPase pre-sequence fused with chloramphenicol acetyltransferase (CAT) is targeted specifically to mitochondria in vivo in transgenic plants. The above demonstrate the specificity of targeting to the chloroplast and mitochondria in plants; only in a few exceptional cases can a protein target both to the chloroplast and mitochondrion

Section 1:7 Proteolytic Processing Of Imported Precursor
Proteins

Section 1:7:a Stromal Processing Peptidase

The pre-sequences of nuclear-encoded chloroplast proteins are proteolytically removed by specific proteases. Those precursors targeted to the stroma or thylakoid membrane have their ETDs cleaved by a stromal processing peptidase (SPP). SPP has been partially purified from Pisum sativum, has an estimated relative molecular mass of 180,000, and a pH optimum of 9 (Robinson & Ellis, 1984). This SPP can recognise a range of chloroplast targeted precursors from a variety of species, suggesting the existence of a single SPP, but as the peptidase has only been partially purified this has not been formally proved (Robinson & Ellis, 1984; per. com. Robinson, C.). As already discussed there does not appear to be a conserved SPP cleavage site at the primary sequence level. However, it is probable that there may be a conserved secondary or tertiary structure which is recognised by SPP. Huang et al., (1990) have provided some preliminary evidence suggesting that the proteases responsible for precursor maturation in mitochondria and

chloroplast targeting are mechanistically similar in their ability to process the pre-sequence of the cytochrome oxidase subunit Va.

Section 1:7:b Thylakoidal Processing Peptidase

Hageman et al., (1986) and James et al., (1989) have shown that precursors which are targeted to the thylakoid lumen can be processed by SPP to a polypeptide that is intermediate in size between the precursor and mature polypeptides. An intermediate of the same size is also observed on import of pre-PC, pre-23K and pre-33K into isolated chloroplasts (James et al., 1989; Hageman et al., 1990). The maturation of this intermediate to a mature sized polypeptide is undertaken by a second peptidase. This peptidase was initially identified by Hageman et al., (1986) using triton X100 solubilized thylakoids and was termed thylakoidal processing peptidase (TPP). A partially purified TPP has been extracted from P.sativum thylakoids; it has a pH optimum of 6.5-7 (Kirwin et al., 1987). The TPP is an integral thylakoid membrane protein with its active site located on the luminal side of the thylakoid membrane (Kirwin et al., 1988). TPP is associated with the thylakoid membrane but does not appear to be associated with any of the thylakoidal complexes; it is exclusively located in the non-appressed lamellae of the thylakoid network (Kirwin et al., 1988). This TPP will cleave the TTDs of a range of precursors from a variety of species, suggesting the existence of a single TPP (Hageman et al., 1986; per. com. Robinson, C.). It is interesting to note that TPP will also cleave the leader peptides of proteins which are targeted

across the endoplasmic reticulum (ER) and the bacterial plasma membrane (Halpin et al., 1989). Sequence analysis of TTDs and leader peptides indicates that small hydrophobic residues tend to be located at the -3 and -1 residues. This -3 X -1 site is thought to be involved in the TPP cleavage event (Hageman et al., 1990). Work on the lut-1 mutation in tobacco suggests that TPP cleavage is not required for the targeting of pre-23K and pre-33K to the thylakoid lumen (Chia et al., 1986). The lut-1 mutation results in the accumulation of both stromal intermediate forms of 23K and 33K (int-23K and int-33K) on the luminal side of the thylakoid membrane. These intermediate forms do not associate with PSII as the mature polypeptides does. It was not shown that the accumulation of the intermediate was due to the failure of the TPP processing event or some other effect of the lut-1 mutation, such as correct association with a thylakoid membrane receptor. The 16kDa polypeptide of the OEC which is nuclear encoded and targeted to the thylakoid lumen was processed to its mature size in the lut-1 mutant, possibly suggesting the existence of two or more TPPs.

Section 1:8 Targeting Of Foreign Proteins

Section 1:8:a Retargeting Using The Envelope Transfer Domain

The pre-sequences of nuclear-encoded precursors which are targeted to the stroma have been shown to contain all of the necessary stroma-targeting information, in that they are able to target foreign proteins across the chloroplast envelope. This was first shown by van den Broeck et al. (1985) who successfully

targeted bacterial neomycin phosphotransferase II across the chloroplast envelope using the pre-sequence of the nuclear-encoded stromal located small subunit of pea rubisco. This first example of the ability of ETDs to target foreign proteins across the chloroplast envelope was in an in vivo situation using transgenic tomato plants. ETDs can also target foreign proteins in vitro into isolated chloroplasts, as seen by the ability of the pre-sequence of the pea small subunit of rubisco to target the coat protein of brome mosaic virus (BMV) into the stroma (Lubben et al., 1989). When Lubben et al. (1989) used the complete precursor of the small subunit of pea rubisco to target the coat protein of BMV, it was found that this could not be targeted across the chloroplast envelope. This indicates that the mere presence of a N-terminal ETD does not always result in targeting across the chloroplast envelope. It is the resultant secondary and tertiary structures of the pre-sequence and mature protein that determine the ability of the mature protein to be targeted across the chloroplast envelope. This point is also seen in the work of Kavanagh et al. (1988) who used the ETD and various amounts of mature chlorophyll a/b binding protein to target the E.coli beta-glucuronidase protein into the chloroplasts of transgenic tobacco plants. It was found that the inclusion of sections of mature chlorophyll a/b binding protein was essential for efficient targeting across the chloroplast envelope.

Section 1:8:b Retargeting Using The Thylakoid Transfer Domain

The pre-sequences of nuclear-encoded thylakoid lumen

proteins can also target foreign proteins across the chloroplast envelope and the thylakoid membrane. This has been shown by the targeting of mouse dihydrofolate reductase (DHFR) into the lumen using the pre-sequences of 33K of wheat and Arabidopsis thaliana (Ko & Cashmore, 1989; Meadows et al., 1989). Ko&Cashmore, (1989) also targeted glycollate oxidase (GO) into the chloroplast using the pre-sequence of 33K. In this case, however, 33K-GO chimeric protein was imported into the stromal compartment and processed to an intermediate form, but it was not further targeted across the thylakoid membrane. This ability of foreign proteins to cross the chloroplast envelope but not the thylakoid membrane was also seen in the in vitro targeting by the S.pratensis plastocyanin pre-sequence of the superoxide dismutase (SOD) protein into chloroplasts (Smeekens et al., 1987). The SOD protein could only be targeted as far as the stromal compartment, although the plastocyanin pre-sequence can target other foreign proteins to the thylakoid lumen with low efficiency (Smeekens et al., 1986; Hageman et al., 1990). The inability of the TTDs to target foreign proteins efficiently to the thylakoid lumen might suggest that the mature protein may contain some targeting information. Evidence for this has come from the expression in tomato plants of a stromal pre-sequence, that of ferredoxin, linked to the mature thylakoid lumen protein, plastocyanin. In the in vivo expression of this chimeric construct, plastocyanin was observed in the thylakoid lumen as a mature sized protein (de Boer et al., 1988). As this chimeric protein only contained a ETD in the pre-sequence the mature plastocyanin must be targeting itself to the thylakoid lumen. It should be noted that when this ferredoxin / plastocyanin chimeric protein was imported in vitro into isolated

chloroplasts, the plastocyanin was only targeted as far as the stromal compartment (Smeekens et al., 1986). Clearly care must be taken when comparing in vivo expression in transgenic plants with in vitro uptake into isolated chloroplasts.

Section 1:8:c The Plastid Family

From the studies on the in vivo expression in tomato plants of the S.pratensis plastocyanin precursor it was shown that the precursor could be targeted into a number of morphologically and functionally differentiated plastids (de Boer et al., 1988). This clearly indicates that the plastid family shares a common import mechanism. Support for this has come from the successful in vitro import of the precursor of the pea small subunit of rubisco into isolated castor oil seed leucoplasts (Boyle et al., 1986). A shared plastid import mechanism is also shown by the ability of the amyloplast waxy precursor to recognise and import successfully into isolated chloroplasts as well as amyloplasts (Klosgen et al., 1989). The waxy polypeptide codes for the UDP-glucose starch glycosyl transferase and catalyzes the synthesis of starch; it is nuclear encoded and targeted to the amyloplast by virtue of an N-terminal targeting signal (Klosgen et al., 1989).

Section 1:8:d Summary

Foreign proteins can successfully be targeted across the chloroplast envelope and the thylakoid membrane by the use of

chloroplast pre-sequences. The chloroplast envelope appears to be more tolerant of the foreign protein that can be targeted across it than does the thylakoid membrane. The ETD would appear to contain all of the essential targeting information required for efficient targeting across the chloroplast envelope. It is not clear whether the TTD contains all of the targeting information required to direct efficient transport across the thylakoid membrane. It may be that the mature protein, at least in some cases, contains some targeting information. It would appear that the ability of a foreign protein to cross the chloroplast envelope or the thylakoid membrane is not solely determined by the presence of ETD or TTD but also depends on the secondary and tertiary structures adopted by each chimeric protein.

Section 1:9 Components Involved In Chloroplast Protein
 Transport

Section 1:9:a Chloroplast Envelope Components

Little is known about the components involved in protein targeting across either the chloroplast envelope or the thylakoid membrane. It would appear from the successful targeting of E.coli expressed Silene pratensis ferredoxin into isolated chloroplasts that no additional cytoplasmic factors are required for targeting across the chloroplast envelope (Pilon et al., 1990). It should be noted that this E.coli expressed ferredoxin was isolated from an insoluble form using 4M urea, and that 0.2M urea was present in the in vitro chloroplast uptake used to assay import. It would then be expected that at a concentration of 0.2M urea the

ferredoxin would be partially unfolded, and could in this partially unfolded state bypass a cytoplasmic or envelope unfoldase activity.

Chloroplast targeted precursors were shown by Pfisterer et al. (1982) to bind tightly to the chloroplast envelope in an ATP-independent manner. This binding was shown by Cline et al. (1985) to be in part due to an interaction with a protein or a number of proteins located in the outer membrane of the chloroplast envelope. This was shown by the protease treatment of isolated chloroplasts with thermolysin. Thermolysin had previously been shown to affect only proteins located in the outer membrane of the chloroplast envelope (Cline et al., 1984). Precursors could be bound to the outside of the chloroplast envelope when the photophosphorylation uncoupler nigericin was present in the import assay; this bound precursor could then be imported into chloroplasts if excess ATP was added. There was a 50% reduction in bound precursor when the chloroplasts were pretreated with thermolysin before being used in an import assay in the presence of nigericin. The above work implicates a proteinaceous receptor in the targeting of precursors across the chloroplast envelope. An outer envelope proteinaceous receptor has also been implicated in the targeting of the inner envelope membrane phosphate translocator, which also fails to target into isolated chloroplasts which have been protease treated (Flugge et al., 1989). The targeting of the outer envelope 6.7kDa polypeptide to the outer envelope is insensitive to protease pretreatment of chloroplasts and will insert into protease treated chloroplasts (Salomon et al., 1990), indicating that a protein receptor is not required for the targeting of the 6.7kDa polypeptide.

A number of attempts have been made to identify the receptor involved in targeting across the chloroplast envelope. Cornwell et al., (1987) used a photoactivatable cross-linker attached to a reagent that reacts with free sulphydryl groups. This heterobifunctional photoactivatable cross-linker was first attached to the precursor of pea small subunit of rubisco via the precursors' free sulphydryl groups. This modified precursor was then bound to the chloroplasts, and was cross linked to the putative receptor by exposure to light. This method identified a 66kDa protein located on the chloroplast surface as a putative import receptor. Kaderbhai et al., (1988) used a 24 residue synthetic peptide derived from wheat small subunit of rubisco containing photoactivatable cross linking amino acid analogs for their cross-linking studies. When this peptide was bound to chloroplasts and exposed to light, the peptide bound to 52kDa protein and a 30kDa protein. The 52kDa protein was shown to be the large subunit of rubisco and the 30kDa protein was proposed to be the chloroplast envelope 30kDa phosphate translocator. Kaderbhai et al., (1988) suggest that the 30kDa phosphate translocator has an additional role in protein targeting as an import receptor. Pain et al., (1988) also used a synthetic peptide, this time derived from pea small subunit of rubisco. Their peptide was used to raise anti-peptide antibodies and these anti-peptide antibodies were then used to raise a second set of antibodies known as anti-idiotypic antibodies. Within this population of anti-idiotypic antibodies would be antibodies which would recognise the antigenic determinants of a putative rubisco small subunit receptor. These anti-idiotypic antibodies immunoprecipitate a chloroplast envelope receptor-bound precursor

complex, the putative receptor being of 30kDa. These anti-idiotypic antibodies, when used in immunogold electron microscopy, identified contact sites between outer and inner membranes of the chloroplast envelope as possible import sites. The antibodies also reacted with a 52kDa protein in the stroma, most probably the large subunit of rubisco. As was pointed out by Joyard *et al.*, (1988), Pain *et al.*, (1988) did not address the possibility that the 30kDa protein identified as a putative receptor by the anti-idiotypic antibodies was in fact the 30kDa phosphate translocator of the chloroplast envelope. Recent work by Schnell *et al.*, (1990) has resulted in the cloning and sequencing of this putative receptor, and it does show extensive homology with the phosphate translocator. Schnell *et al.*, (1990), however, argue against this putative receptor and phosphate translocator being homologues on the basis of different binding characteristics to hydroxylapatite. The possibility that the putative receptor and the phosphate translocator are one and the same protein which happens to have two activities is dismissed. Schnell *et al.*, (1990) argue against a dual role for the putative receptor on the basis that a receptor would have to be located in the outer envelope whereas the phosphate translocator is located in the inner envelope (Flugge *et al.*, 1989) and antibodies raised to the putative receptor cross react with isolated chloroplasts. However, Pain *et al.*, (1988) and Schneel *et al.*, (1990) have both implicated zones of contact between outer and inner envelope as sites of import, it is thus not inconceivable that a receptor spanning both membranes would be accessible to antibodies raised to the putative receptor.

Deletion analysis of the pre-sequence of the pea small

subunit of rubisco has been used in attempts to identify regions in the pre-sequence important for binding and translocation. It was found that the extreme C- and N- terminal regions as well as a central region of the pre-sequence are important for binding to the putative receptor but not for translocation across the chloroplast envelope (Reiss et al., 1989). It was not possible in this study to pin down any region exclusively involved in protein translocation.

Section 1:9:b Thylakoid Membrane Components

Studies into targeting across the thylakoid membrane have not been possible because of the lack of an in organellar uptake system. Recently Kirwin et al., (1989) have developed an ATP-dependent isolated thylakoid uptake system for the import of luminal proteins, the efficiency of this system is, however, poor. Mould et al., (in press) has developed an efficient light driven thylakoid uptake system for luminal protein which will allow the role of proteinous components to be addressed. The only proteinous component to be implicated in the targeting across the thylakoid membrane is TPP (Chia et al., 1986 & Hageman et al., 1986 & section 1:7:b).

Section 1:9:c The Role Of Lipid In Protein Targeting

The role of the lipid component of the chloroplast and thylakoid membrane in protein targeting has received very little attention. It is thought that chloroplast pre-sequences can form amphiphilic helices, which may partition into lipid bilayers in a

similar manner to mitochondrial pre-sequences. Chloroplast pre-sequences will associate with the lipid bilayers of the outer membrane of the chloroplast envelope and the thylakoid membrane, as was seen when these were protease treated (Cline et al., 1985 & L. Barnett (per. com.). The specificity of protein targeting within a cell may result from the unique lipid content of the chloroplast envelope, mitochondrial envelope, ER and thylakoid membrane. Such specificity has been shown in the mitochondrial system which requires the unique mitochondrial envelope lipid cardiolipin for insertion of mitochondrial pre-sequences into a lipid bilayer to take place (Hartl et al., 1989). The ER system has been shown to require no membrane proteins for the translocation of some proteins (Rapoport, 1990). Acidic phospholipids have also been implicated in an unfolding activity (Endo & Schatz, 1988). Any model for targeting into the chloroplast will have to consider the role of the lipid component of the chloroplast envelope and the thylakoid membrane, unless proteins are targeted across these membranes via a protein pore complex.

Section 1:10 Energy Requirements For Protein Targeting Across
The Chloroplast Envelope And Thylakoid Membrane

Section 1:10:a Energetics Of Proteins Targeted Across The
Chloroplast Envelope

The energetics of protein targeting across the chloroplast envelope and the thylakoid membrane have been studied using the isolated chloroplast and thylakoid assay systems. It has become

clear through the use of uncouplers and inhibitors of the electrical and chemical components of the proton motive force (PMF); that a PMF is not required for the targeting of precursors across the chloroplast envelope (Grossman et al., 1980; Cline et al., 1985; Flugge et al., 1986; Pain et al., 1987; Theg et al., 1989). It would appear that ATP is required for targeting across the chloroplast envelope; the mechanism by which ATP is used to drive import is unknown although a chloroplast ATPase has been implicated in the import mechanism (Pain et al., 1987). This ATPase appears to be located in the stroma and not linked to other ATPase activities shown in the thylakoid membrane or inner envelope membrane. It is not involved in the generation of H^+ , Na^+ , K^+ , Ca^+ or Mg^+ gradients across the inner envelope membrane. The ATPase activity may form part of a translocase or it could be involved in a kinase activity. The ATP required during protein import across the chloroplast envelope may mediate protein import via a phosphorylation/dephosphorylation cycle of the imported precursor or of some component of the import apparatus (Keegstra & Olsen, 1989; Pain et al., 1987). Protein import can be inhibited by the use of phosphatase inhibitors, such as sodium fluoride and sodium molybdate (Soll & Buchanan, 1983; Flugge et al., 1986; Pain et al., 1987). A number of protein kinase activities have also been identified in the outer envelope of the chloroplast membrane (Soll & Buchanan, 1983; Soll et al., 1988). The cAMP-independent protein kinase identified by Soll & Buchanan, (1983) will phosphorylate the small subunit of rubisco after binding to the chloroplast envelope but before its translocation. The phosphorylation of a 51kDa outer envelope protein has been shown to be correlated with the import of the

small subunit of rubisco (Hinz & Flugge, 1988). This 51kDa protein has been suggested to have a role in receptor/precursor binding.

The site of action of ATP has been the subject of some controversy. Flugge *et al.*, (1986) found that ATP was required outside the chloroplast whereas Pain *et al.*, (1987) and Theg *et al.*, (1989) found that ATP was required internally. Keegstra *et al.*, (1989) suggest that the external ATP trap used by Flugge *et al.*, (1986) would also affect the phosphorylation state of imported proteins and this, rather than the removal of external ATP, may have resulted in the failure of precursors to import into chloroplasts. Olsen *et al.*, (1989) have shown that ATP has at least two distinct roles in protein targeting across the chloroplast envelope. ATP is required at a low concentration for the binding of precursors to the chloroplast envelope and at a higher concentration for the translocation of precursors across the envelope. Earlier studies had suggested that ATP was not required for the efficient binding of precursors to the chloroplast envelope (Pfisterer *et al.*, 1982).

Clearly, ATP hydrolysis has a role in the targeting of precursors across the chloroplast envelope, but ATP hydrolysis is also required for the targeting of the phosphate translocator to the inner membrane of the chloroplast envelope (Flugge *et al.*, 1989). In contrast, ATP hydrolysis is not required for the integration of the 6.7kDa polypeptide of the outer membrane of the chloroplast envelope (Salomon *et al.*, 1990). The 6.7kDa polypeptide shows a number of features common to the targeting of apo-cytochrome c (a nuclear-encoded polypeptide that is targeted to the intermembrane space of the mitochondria): (i.) it lacks a

cleavable targeting signal, (ii.) it does not require an exposed surface receptor, (iii.) it does not require ATP hydrolysis for integration (sections 1:6:a; 1:9:a; and Pfanner et al., 1988).

Section 1:10:b Energetics Of Protein Targeting Across The Thylakoid Membrane

Externally added ATP has been shown to enhance import across the thylakoid membrane of pre-33K (Kirwin et al., 1989). Recent work by Mould et al., (1991) has shown that the targeting across the thylakoid membrane of a number of precursors (excluding plastocyanin) requires the proton gradient component but not the electrical potential component of the thylakoidal PMF. In contrast to Mould et al., (1991), Theg et al., (1989) showed that plastocyanin only requires internal ATP and does not require a PMF to cross the thylakoid membrane. Externally added ATP as well as a proton gradient were also required for the efficient integration of LHCP into the thylakoid membrane (Viitanen et al., 1988; Cline et al., 1989).

Section 1:11 Protein Targeting Across Other Membranes

As noted in figure 1, proteins are targeted to a wide variety of compartments. The mechanisms of protein targeting across the mitochondrial envelope, bacterial plasma membrane, E.R. and peroxisome membranes have been studied in some detail. From these studies protein targeting can be divided into a number of common elements: targeting signals, chaperones, receptors, translocators, peptidases and energetics. The following sections

will consider targeting across a variety of membrane systems and their relevance to targeting across the chloroplast envelope and the thylakoid membrane.

Section 1:11:a Targeting To The Mitochondria

The mitochondrion, like the chloroplast is bounded by a double envelope membrane but unlike the chloroplast does not contain an internal membranous network equivalent to the thylakoid network. Mitochondria also resemble chloroplasts by containing their own genome which encodes a small number of proteins; the vast majority of mitochondrial proteins are nuclear-encoded and are synthesised in the cytosol. Most of these nuclear-encoded proteins are synthesised with N-terminal pre-sequences which are responsible for targeting into the mitochondria, although a number of polypeptides appear to have internal targeting signals such as porin (targeted to the outer envelope), cytochrome c (targeted to the intermembrane space), ADP/ATP carrier (targeted to the inner envelope) and 2-isopropyl malate synthetase (targeted to the matrix) (Hartl et al., 1989). Mitochondrial proteins, such as cytochrome b_2 which are targeted to the intermembrane via the matrix appear to have a two step import mechanism similar in many ways to that seen for targeting of nuclear-encoded thylakoid lumen polypeptides; in that the N-terminal domain of the mitochondrial targeting signal first targets the precursor of cytochrome b_2 across the mitochondrial envelope where it is cleaved by the matrix processing peptidase, the C-terminal section of the targeting signal then retargets the matrix intermediate across the inner mitochondrial envelope

membrane to the intermembrane space where the inner matrix processing peptidase (IMP) cleaves the intermediate to a mature polypeptide (Hartl et al., 1987).

Section 1:11:a:i Mitochondrial Targeting Signals

The N-terminal targeting signals of both mitochondrial and chloroplastic precursors lack extensive hydrophobic regions, are rich in basic and hydroxylated residues, contain few acidic residues (Verner and Schatz, 1988) and are both termed "hydrophilic" pre-sequences. Von Heijne et al., (1989) showed that the hydrophilic pre-sequences of mitochondrial and chloroplast proteins can be distinguished by their abilities to form amphiphilic alpha helices. The central region of mitochondrial targeting signals can form amphiphilic alpha helices which can interact with lipid bilayers (Roise et al., 1986; Tamm, 1986; Roise et al., 1988; von Heijne et al., 1989). Only a small C-terminal section of the chloroplast envelope targeting signal (ETD) can be folded into an amphiphilic beta sheet (von Heijne et al., 1989). This beta sheet would seem by its position and size to be important for processing by SPP and not for insertion into lipid bilayers (von Heijne et al., 1989). The above analysis was based on the comparison of higher plant ETDs with mitochondrial targeting signals. A comparison of the green alga C.reinhardtii ETDs with mitochondrial targeting signals has revealed that these signals are more similar, and both can be folded into amphiphilic alpha helices (Franzen et al., 1990). This similarity may provide an explanation for the ability of the pre-sequence of C.reinhardtii rubisco SSU to

re-target DHFR into yeast mitochondria (Hurt et al., 1986).

The N-terminal pre-sequences of a number of intermembrane space polypeptides are bipartite in nature consisting of a hydrophilic N-terminal targeting signal followed by a hydrophobic targeting signal. These bipartite mitochondrial pre-sequences resemble the bipartite targeting signals for nuclear-encoded thylakoid lumen polypeptides (Hartl et al., 1989; section 1:6:c). Analysis of the cleavage sites of these mitochondrial bipartite pre-sequences and the cleavage sites of thylakoid lumen precursors indicate that both contain small hydrophobic residues at the -3 and -1 positions, suggesting a possible functional similarity between the mitochondrial IMP and TPP (Kaput et al., 1983; Sadler et al., 1984 and Guiard, 1985).

Section 1:11:a:ii Proteases Involved In the Cleavage Of Mitochondrial Targeting

The N-terminal pre-sequences of mitochondrial targeted precursors are cleaved in part or in total by a protease located in the matrix. This matrix processing peptidase (MPP) is a 57kDa polypeptide in Neurospora crassa and its activity is enhanced by a 52kDa polypeptide known as the processing enhancing protein (PEP)(Hawlitsek et al., 1988). MPP shows activity in the absence of detergents and at a neutral pH, it is stimulated by divalent cations and inactivated by EDTA and ortho-phenanthroline (Witte et al., 1988; Hartl et al., 1989). These properties are shared with SPP which removes the N-terminal pre-sequences of precursors targeted across the chloroplast envelope (section 1:7:a). The two proteases can not recognise each others

precursors as judged by in vitro processing assays (per. com. C.Robinson), suggesting that although they carry out similar functions they recognise different cleavage signals. However, recent work by Huang et al., (1990) suggests that a mitochondrial pre-sequence can recognise and target a protein across the chloroplast envelope into the stroma where the mitochondrial pre-sequence is cleaved by SPP; this cleavage by SPP occurs when the mitochondrial chimeric protein is expressed in vivo in transgenic plants (section 1:6:d). Clearly this raises the possibility that MPP and SPP can recognise the same cleavage motif in some cases.

The peptidase responsible for the second cleavage event in the two step targeting of inter membrane space polypeptides such as cytochrome b_2 (cytb₂) has been identified as being composed of 21.4kDa subunits (Hartl et al., 1987; Schneider et al., 1991). The inner membrane protease I (IMPI) is an integral inner membrane protease with its active site on the trans-side of the membrane and is active in detergent extracts (Schneider et al., 1991). These characteristic features of the IMPI are also shared by TPP, which is responsible for the removal of the TTD of polypeptides targeted across the thylakoid membrane (section 1:7:b; per.com. C.Robinson).

Section 1:11:a:iii Energetics Of Protein Targeting Into Mitochondria

In most systems the driving force for protein targeting appears to be ATP (or GTP) and sometimes the electrical or chemical components of the proton motive force (PMF)(Eilers and Schatz, 1988). In mitochondrial protein targeting there is a

requirement for ATP on the cis-side of the mitochondrial membrane (outside of the inner envelope)(Hartl et al., 1989). This ATP requirement is similar to the ATP requirements of protein targeting across the chloroplast envelope, although ATP is also required on the trans-side of the chloroplast envelope. Protein targeting across the mitochondrial envelope also requires the electrical component of the PMF whereas protein targeting across the chloroplast envelope does not require a PMF (Eilers et al., 1987; Pfanner et al., 1988b; section 1:10:a). The electrical component of the PMF is only required in the early stages of protein targeting into the mitochondria, one of its possible modes of action has been suggested as an involvement in an electrophoretic effect between the pre-sequence and the membrane (Hartl et al., 1989). The cis requirement for ATP in mitochondrial import appears to have a role in the unfolding and the maintenance of import competence of the precursor (Hartl et al., 1989). The cis requirement for ATP in chloroplast targeting could possibly have a similar role (section 1:12:b).

Section 1:11:b The Export Of Proteins From Escherichia coli

The export of proteins from E.coli represents the best studied example of a protein targeting system. The combined approaches of genetic, biochemical and physiological analysis possible with E.coli has lead to a basic understanding of protein export. Genetic analysis has led to the identification of a number of sec alleles which represent cells exhibiting severe protein export defects under nonpermissive conditions and pri

alleles which represent cells which are able to restore protein export of proteins with defective targeting signals. Most of the export mutants have been cloned, sequenced and functional proteins have been isolated and reconstituted into translocation-competent proteoliposomes.

Based on a variety of biochemical and genetic studies reviewed by Bassford et al., (1991) translocation can occur co- or post-translationally, the precursor first forms a cytoplasmic complex with the SecB protein (chaperone) thus preventing misfolding of the precursor. This SecB/precursor complex then binds to the membrane via an interaction with SecA (PrID). The peripheral cytoplasmic membrane protein SecA interacts with the integral membrane protein SecY (PrIA) and SecE (PrIG). SecA has an ATPase activity which along with a PMF is required for translocation across the membrane. SecB and SecF act late in the translocation step and appear to have a role in the release of proteins after translocation. At least two peptidases have been identified which cleave proteins exported from E.coli, leader peptidase (LEP) and lipoprotein signal peptidase (LSP) (section 1:11:b:ii). Recently E.coli proteins Ffh and FtsY and 4.5S RNA species (FSs) have been identified that exhibit sequence similarities to signal recognition particle (SRP) and docking protein (DP) from the E.R. targeting system (Bassford et al., 1991). It remains unclear what the role if any these proteins have in E.coli export.

The following sections will highlight those areas of similarity between E.coli export and chloroplast protein import.

Section 1:11:b:i The role Of Targeting Signals In Escherichia

coli Export

A number of E.coli proteins such as the maltose binding protein are targeted from the cytosol across the cytoplasmic membrane into the periplasmic space by virtue of a hydrophobic N-terminal pre-sequence (Lazdunski and Benedetti, 1990). These targeting signals share a number of common features with TTDs: an N-terminal +ve charge, a central hydrophobic domain and small hydrophobic residues located at the -3 and -1 positions (von Heijne, 1985; von Heijne et al., 1989; Franze et al., 1990).

Section 1:11:b:ii Proteases Involved In Escherichia coli Export

Most of the bacterial signal sequences are cleaved by the 36kDa leader peptidase (LEP) on the trans-side of the cytoplasmic membrane (Date & Wickner 1981; Wolfe et al., 1983). LEP is an integral membrane protein with its active site located on the trans-side of the cytoplasmic membrane; the isolated enzyme is active only in the presence of detergents (Zimmermann et al., 1982; Wolfe et al., 1983). These features of LEP are similar to those of TPP (section 1:7:b). The presence of small hydrophobic residues immediately before the peptidase cleavage site (the -3 X -1 site) of both bacterial signal sequences and TTDs may indicate that LEP and TPP recognise the same or similar cleavage signals. Halpin et al., (1989) showed that LEP and TPP will recognise the cleavage sites of precursors that are targeted across the thylakoid and bacterial plasma membranes; indicating that these proteases are mechanistically related. Antibodies raised to LEP do not recognise TPP suggesting the two peptidase are not

identical (Halpin et al., 1989). The above processing experiments indicate that LEP will process nuclear encoded thylakoid lumen proteins in vitro. When the nuclear encoded thylakoid lumen pre-33K or a truncated 33kDa OEC precursor lacking the ETD were expressed in E.coli., both precursors were targeted across the plasma membrane and processed by LEP to a mature sized product (Seidler & Michel, 1990; Meadows & Robinson, in press). This indicates that not only are TPP and LEP mechanistically related, but that the mechanisms of transport across the thylakoid and bacterial plasma membranes are also mechanistically related. It was not shown in either of the above that the export across the plasma membrane was secA / secY dependent. The similarity of thylakoid import to E.coli export will allow useful comparisons to be drawn between the well characterised E.coli system and the little studied thylakoid system.

At least one other prokaryotic signal peptidase has been identified. Tokunaga et al., (1982) have shown that antibodies raised to LEP (procoat signal peptidase) prevented cleavage of procoat protein but did not affect cleavage of prolipoprotein, clearly indicating the existence of at least two prokaryotic signal peptidases.

Section 1:11:b:iii The Energetics Of Escherichia coli Export

The efficient targeting of proteins across the E.coli cytoplasmic membrane requires both ATP on the cis-side of the membrane as well as the electrical gradient and proton gradient of the PMF (Geller et al., 1986; Romisch et al., 1987). The energetics of polypeptide targeting across the E.coli cytoplasmic

membrane show no similarity to those of targeting across the chloroplast envelope (section 1:10:a). The energetics of targeting across the thylakoid membrane are similar to those of E.coli in respect of their requirement for a PMF across the membrane. However, targeting across the thylakoid membrane only requires the proton gradient of the PMF and does not need the electrical potential (section 1:10:b).

Section 1:11:c Protein Targeting Across The Endoplasmic Reticulum Membrane

The targeting of proteins across the endoplasmic reticulum (E.R.) membrane has been studied extensively (recently reviewed by Rapoport, (1990)); this section will outline the basic mechanism of targeting across the E.R. membrane and highlight those areas where a link has been shown between the E.R. and chloroplast targeting systems.

A characteristic feature of protein targeting across the E.R. membrane is an N-terminal signal sequence which is proteolytically removed after translocation across the membrane. As the protein is synthesised on the ribosome the cytosolic signal recognition particle (SRP) binds to the signal sequence resulting in an arrest of translational elongation. SRP is a ribonucleoprotein complex composed of six distinct polypeptide chain and a 300-nucleotide RNA molecule. SRP not only acts to slow translational elongation but also appears to have an antifolding function as well (acting as a molecular chaperone). The SRP / signal sequence / ribosome / mRNA complex then associates with the E.R. membrane via the binding of SRP with its

receptor (SRP-receptor or docking protein) and an association of the ribosomes with two integral membrane proteins ribophorins I and II. After docking of the complex is completed, both the signal sequence and the ribosome are released from SRP and translation elongation, and translational elongation can continue. The signal sequence itself also appears to interact directly with an integral 34kDa-35kDa glycoprotein termed signal sequence receptor (SSR). The role of SSR is unclear but it could form part of a proteinaceous channel for the translocation of proteins. The mechanism of translocation is unclear and may occur via a proteinous tunnel or by passage directly through the hydrophobic phospholipid bilayer. After targeting across the E.R. membrane the signal sequence is cleaved by an integral membrane protein, signal peptidase (SP). The active SP exists as a complex of six polypeptides which also require phospholipids for biological activities.

Section 1:11:c:i The Role of Targeting Signals In Targeting
Across The E.R. Membrane

The signal sequence is responsible for the targeting of proteins across the E.R. membrane like TTDs are hydrophobic targeting signals consisting of a hydrophilic N-terminal region, followed by a hydrophobic core region and ending with a hydrophilic C-terminal region (Verner and Schatz, 1988; von Heijne et al., 1989). The -3 and -1 positions of the eukaryotic signal tend to be small hydrophobic residues as was the case for TTDs (section 1:6:c; von Heijne, 1985).

Section 1:11:c:ii Proteases Involved In E.R. Targeting

The similarity of the region immediately before the eukaryotic signal peptidase cleavage site (-3 and -1 residues) to the region immediately before the TPP cleavage site indicates that these peptidases may well recognise similar cleavage sites. Halpin et al., (1989) have provided some direct data that this may well be the case with the ability of TPP to recognise the targeting signal of pre-pro-alpha factor and cleave it to its mature size.

Section 1:11:c:iii The Energetics Of Protein Targeting Across The E.R. Membrane

Protein targeting across the E.R. requires ATP but does not require a PMF (Rothblatt and Meyer, 1986; Walters and Blobel, 1986; Eilers and Schatz, 1988). In this respect it shows similarities to protein targeting across the chloroplast envelope but not the thylakoid membrane (section 1:10:a and 1:10:b).

Section 1:12 Summary

The following section will attempt to draw the common characteristics of protein targeting together, and give an over view of protein targeting.

Section 1:12:a Targeting Signals

Protein targeting across the mitochondrial envelope, ER,

bacterial plasma, chloroplast envelope and the thylakoid membrane is often mediated by an N-terminal cleavable pre-sequence. These pre-sequences fall into two major groups. The first "hydrophobic" group consist of a hydrophilic N-terminal region, followed by a hydrophobic core region and ending with a hydrophilic C-terminal region (Verner & Schatz, 1988; von Heijne et al., 1989). In this group fall the targeting signals of ER, bacterial and TTD proteins. The second "hydrophilic" group lack extensive hydrophobic regions, are rich in basic and hydroxylated residues, and contain few acidic residues (Verner & Schatz, 1988; von Heijne et al., 1989). In this second group fall the chloroplast and mitochondrial targeting signals.

The hydrophilic targeting signals for the chloroplast and mitochondrial envelope membranes may be distinguished by their ability to form amphiphilic alpha helixes (von Heijne et al., 1989). Mitochondria and chloroplast envelope membrane targeting signals, although sharing some common features, do not appear to use the same import mechanism. This has been confirmed by the observation that chloroplast precursors are not imported into mitochondria (with the exception of C.reinhardtii signals) and mitochondrial precursors do not generally target into chloroplasts (Smeekens et al., 1987; Whelan et al., 1990). This specificity of targeting could be provided by a receptor / precursor interaction, and other import components may be more similar. It has been suggested that the mistargeting of C.reinhardtii small subunit of rubisco into mitochondria results from the bypassing of the receptor step (Hartl et al., 1989).

Von Heijne et al., (1985) have been able to develop computer programmes that can distinguish between the hydrophobic

targeting signal required for E.coli export and targeting across the E.R. Sequence analysis of targeting signals required to cross the thylakoid, bacterial plasma membrane and ER has revealed a number of common features: an N-terminal +ve charge, a central hydrophobic domain and small hydrophobic residues at the -3 and -1 positions (von Heijne, 1985; von Heijne et al., 1989; Franzen et al., 1990). Halpin et al., (1989) have shown that the targeting signals of these three systems can all be recognised and cleaved by peptidases from the thylakoid and E.coli systems, suggesting a shared cleavage mechanism. In support of these results Meadows & Robinson, (in press) and Seidler & Michel, (1990) have also demonstrated that the TTD can act as a targeting signal in E.coli export, the TTD being cleaved by a prokaryotic signal peptidase.

Section 1:12:b The Role Of Chaperones In Protein Targeting

The chaperones are a family of unrelated proteins that mediate the correct folding (assembly) of other polypeptides, but are not components of the mature structure (Ellis, 1990). A role for chaperones has been implicated in protein targeting across the chloroplast envelope, mitochondrial envelope, bacterial plasma membrane and E.R. membrane. The relationship of these various chaperones remains unclear at the moment, but the chaperones so far identified appear to be involved in an ATP dependent unfoldase activity required to make the precursor translocation-competent.

Section 1:12:c Receptor Interaction

A proteinaceous membrane receptor required for the binding of a precursor to the target membrane has been implicated in most protein targeting systems. In the mitochondrial system there are receptors that bind families of precursor (MOM 19 and MOM 72 (mitochondrial outer membrane proteins of 19kDa and 72kDa)), these precursor / receptor complexes then interact with one common component, the general insertion protein (GIP) (Pfanner et al., 1991). In the E.R. system the membrane bound receptor is signal sequence receptor (SSR) (Lazdunski & Benedetti, 1990), whereas in the bacterial system there is some evidence for sec Y being a membrane bound receptor (Akiyama & Ito, 1987). In chloroplasts a 30kDa polypeptide is a promising candidate for the envelope receptor (Pain et al., 1988; Schnell et al., 1990).

Section 1:12:d Translocation Mechanism

Little is known about the mechanism of translocation across a membrane, but it appears that translocation can occur post-translationally in all systems (Lazdunski & Benedetti, 1990). The debate whether protein translocation occurs via a protein pore or by direct interaction with the lipid bilayer has not been resolved (Lazdunski & Benedetti, 1991), but appears to favour a protein translocator. In bacterial export, E.R. targeting and mitochondrial targeting putative components of the translocator have been isolated (Lazdunski & Benedetti, 1991). It is also generally agreed that protein translocation occurs in an unfolded state. Hartl et al., (1989); Pain et al., (1988) and Schnell et al., (1990) have shown that protein targeting across the mitochondrial envelope and chloroplast envelope occur at points

of contact between the inner and outer membrane of their respective envelopes.

Section 1:12:e Processing Of Targeting Signals

N-terminal targeting signals are cleaved by specific peptidases located on the trans-side of their respective membranes. Hydrophobic targeting signals are cleaved by integral membrane proteins, such as E.coli leader peptidase (LEP) (Wolfe et al., 1983), eukaryotic signal peptidase (SP). Hydrophilic targeting signals are cleaved by soluble metalloproteases such as mitochondrial matrix processing peptidase (MPP)(Hartl et al., 1989) and stromal processing peptidase (SPP)(Robinson and Ellis, 1984).

Section 1:12:f Energetic Requirements

ATP appears to be required by all protein targeting systems. Its exact role has not been determined, but it appears to be required for the unfolding of precursors prior to translocation (Lazdunski & Benedetti, 1990). Some systems also require a PMF; in bacteria both the electrical gradient and chemical gradient components of the PMF are required (Geller et al., 1986; Romisch et al., 1987). The mitochondrial envelope system only requires the electrical component of the PMF (Eilers et al., 1987; Pfanner et al., 1988), whereas the thylakoid membrane system requires the chemical component of the proton gradient (Mould & Robinson, 1991). The PMF is not required for targeting across the chloroplast envelope, E.R. and peroxisome membrane (Grossman et

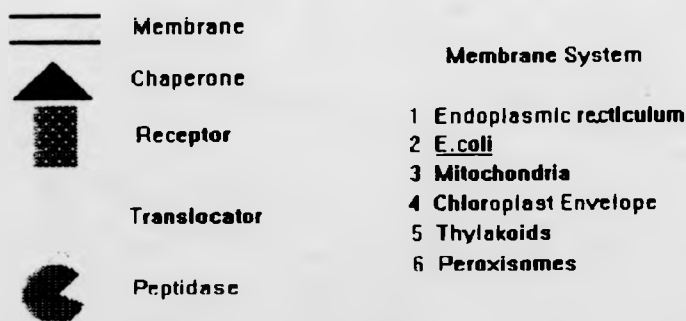
al., 1980; Baker and Randal, 1984; Pfanner et al; 1985; Hansen et al; 1986; Rothblotl and Meyer. 1986; Walters and Blobel, 1986).

Section 1:12:g The Unified Model Of Protein Targeting

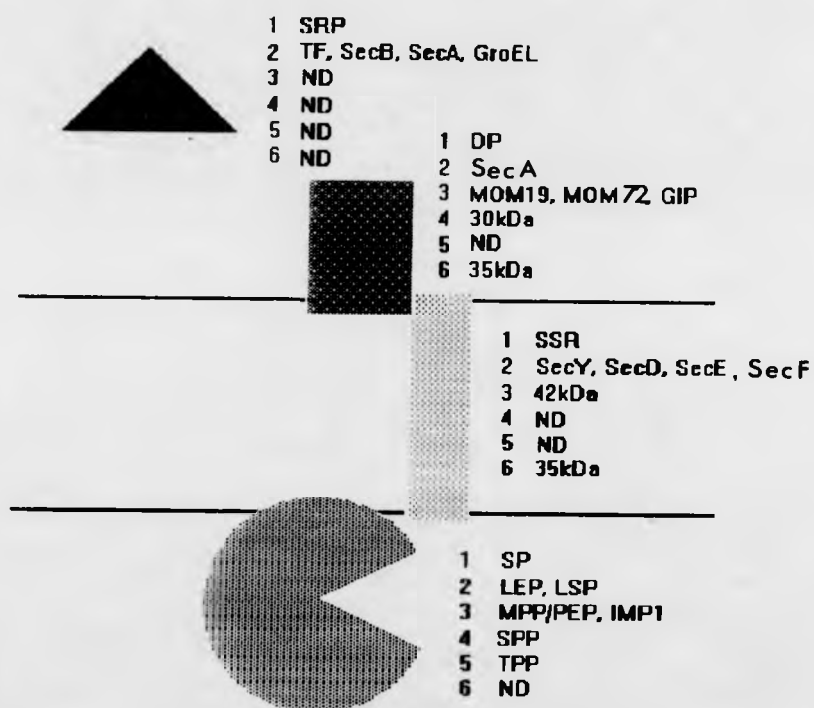
Based on the above work it appears that in general proteins with N-terminal pre-sequences which are targeted across a membrane first interact with a chaperone which unfolds the precursor, in an ATP dependent step. This translocation competent precursor then binds to a receptor in a PMF dependent step in some systems. This receptor bound precursor then translocates across the membrane either by direct integration with the lipid bilayer or via a protein translocator. After the translocation the targeting signal is removed by a specific protease. Figure 9 summarises the possible components involved in protein targeting from the various systems.

Figure 9 A schematic representation of the various proteineous import components required for targeting across a membrane

Based on the studies of protein targeting across the endoplasmic recticulum, periplasmic membrane and mitochondrial envelope it would appear that four classes of protein are required for targeting across a membrane: a chaperone, receptor, translocase and peptidase. These components, where known, are indicated.



SRP Signal recognition particle
 TF Trigger factor
 DP Docking protein
 SSR Signal sequence receptor
 SP Signal peptidase
 LEP Leader peptidase
 LSP Prolipoprotein signal peptidase
 MPP Matrix processing peptidase
 PEP Processing enhancing protein
 SPP Stromal processing peptidase
 TPP Thylakoidal processing peptidase
 ND No data



SECTION II- MATERIALS AND METHODS

Section 2:1 Preparation And Running Of An Agarose Gel

50ml of 0.4% (w/v) agarose in 0.5xTBE (0.045M Tris-borate and 0.01M EDTA) was microwaved until all the agarose had dissolved. This mixture was allowed to cool to 60°C before ethidium bromide was added to a final concentration of 0.5µg/ml and then the mixture was cast in a BRL "Horizon 58" horizontal slab gel and a slot-former was inserted. When the gel was set the slot-former was removed and the DNA samples were loaded after being mixed with 0.5 volumes of DNA loading buffer (0.25% bromophenol blue and 40% (w/v) sucrose in water). The gel was run in 0.5xTBE according to the manufacturers instructions. The DNA bands were visualized using a u.v. transilluminator.

Section 2:2 Preparation And Running Of A DNA Polyacrylamide Gel

A 10% polyacrylamide slab gel (16.5x14x0.1cm) was used to resolve DNA fragments below 500bp. 30ml of acrylamide was made up as below:

10xTBE	3ml
30%(w/v) acrylamide / 0.8%(w/v) bisacrylamide	10ml
50%(v/v) glycerol	3ml
H ₂ O	14ml
TEMED	144µl
10% (w/v) ammonium persulphate	193µl

This mixture was then poured into the gel, a slot-former was inserted, and this was allowed to polymerise. After polymerisation the slot-former was removed and the gel mounted in the electrophoresis tank (Scie-Plas). The running buffer was 1xTBE (0.09M Tris-borate and 0.02M EDTA). The DNA samples were loaded after being mixed with 0.5 volumes of DNA loading buffer (0.25% bromophenol blue and 40% (w/v) sucrose in water). The gel was run in 1xTBE at 40mA constant current. After the gel had been run it was submerged in 1xTBE containing 0.5µg/ml ethidium bromide for 30mins. This stained gel was visualized using an u.v. transilluminator.

Section 2:3 Phenol / Chloroform Extractions Of DNA

The DNA containing solution was mixed with 0.5 volumes of phenol/chloroform (1:1) and left to stand for 15mins at room temperature. The mixture was centrifuged at high speed in the bench top microfuge for 5mins at room temperature. The upper aqueous phase was removed and mixed with 0.5 volumes of chloroform. This mixture was centrifuged at high speed in the bench top microfuge for 5mins at room temperature. The upper aqueous phase was removed and mixed with 0.1 volumes of 3M Na acetate pH5.6. This mixture was either mixed with 1 volume of isopropanol or 2.5 volumes of ethanol. The solvent used depends on the volume of the starting solution. The DNA was precipitated by freezing the above solution in an ethanol/dry ice bath. The precipitated DNA was pelleted by centrifugation at high speed in

the bench top microfuge for 15mins. The pelleted DNA was dried under vacuum and resuspended in H₂O

Section 2:4 Extraction Of DNA Fragments From A Polyacrylamide Gel

DNA previously digested with appropriate restriction enzymes was electrophoresed in a polyacrylamide gel and stained with ethidium bromide as discussed in section 2:3. The stained polyacrylamide gel was viewed on an u.v. transilluminator and the DNA band to be extracted was excised. This band was electroeluted into 10mM Tris-Cl pH 7.2, 10mM EDTA pH 7.2 (TE) buffer using the, "Biotrap" (Schleicher & Schuell) following the manufacturers instructions. The DNA was phenol/chloroform extracted, ethanol precipitated and dried before being resuspended in H₂O.

Section 2:5 Extraction Of DNA Fragments Using Low Melting Point Agarose

DNA previously digested with appropriate restriction enzymes was electrophoresed in 0.8% w/v low melting point agarose made up in TBE buffer. DNA was electrophoresed until the ethidium bromide stained fragments could be distinguished from each other under u.v. light and then the required fragment was excised from the gel, taking care to remove any excess agarose. The agarose slice containing the DNA fragment was placed into 150µl of TE (pH8.0) buffer at 65°C, a temperature at which low melting point agarose dissolves. The solubilized DNA was phenol/chloroform extracted,

ethanol precipitated and dried. The dried DNA pellet was dissolved in H_2O .

Section 2:6 Treatment Of Linearised Plasmid DNA With Alkaline Phosphatase

Treatment of linearised vector with alkaline phosphate (calf intestinal) removes 5'-terminal phosphate groups thus preventing recircularisation of vector during ligation. The addition of non-phosphatased insert provides a 5'terminal phosphate on one end of each strand between vector and insert. The unligated nick at the opposite end of each strand is repaired by the host cell repair mechanism following transformation of E.coli.

The phosphatase reaction was carried out as follows:-

Restricted vector (approximately 500ng total DNA) was redissolved after ethanol precipitation in CIP buffer (50mM Tris-Cl pH9.0, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 1mM spermidine plus 50mM glycine pH9.4) and incubated with 1 unit of calf intestinal phosphatase (CIP, Bohringer). The reaction mix was incubated for 15mins at 37°C followed by 15mins at 56°C. A further 1 unit of CIP was added and the two stage incubation repeated. The reaction was stopped by dilution with 20 μ l H_2O and addition of 5 μ l 10xTNE (100mM tris-Cl pH8.0, 1M NaCl, 10mM EDTA), followed by heating at 68°C for 15mins. Finally the phosphatased DNA was extracted twice with phenol/chloroform, ethanol precipitated and dried before being resuspended in H_2O .

Section 2:7 Treatment Of Linearised Plasmid Or DNA Inserts
With S1 Nuclease

Treatment of linearised plasmid or DNA inserts with S1 nuclease removes 5' overhangs resulting in blunt ends. The nuclease reaction was carried out as follows.

Restricted vector (approximately 500ng total DNA) was redissolved after ethanol precipitation in 100µl of S1 nuclease buffer (200mM NaCl, 50mM sodium acetate pH4.5, 1mM ZnSO₄ and 0.1% glycerol) and incubated with 20 units of S1 nuclease. The reaction mix was incubated for 30 minutes at 37°C. The reaction was stopped by extraction with phenol / chloroform (50:50) and precipitation in ethanol.

Section 2:8 Ligation Of DNA Fragments

Plasmid or phage DNA (20ng per 10µl ligation) and isolated DNA fragments (100ng per 10µl ligation) were ligated overnight at 15°C in ligase buffer (50mM Tris-Cl pH7.5, 5mM MgCl₂, 5mM DTT, 10mM ATP) with 0.1units/µl of T4 DNA ligase (Amersham). After ligation, competent E.coli cells of an appropriate strain were transformed with the ligation mixture.

Section 2:9 Growth And Maintenance Of Escherichia coli Strains

Stock strains of E.coli were plated on L-Agar media (1%(w/v) tryptone, 0.5%(w/v) yeast extract, 1.5%(w/v) agar, 85mM NaCl) and stored at 4°C. Liquid cultures of E.coli were grown in L-Broth

(1%(w/v) tryptone, 0.5%(w/v) yeast extract, 170mM NaCl). Liquid cultures were maintained at 37°C in orbital shakers at 200rpm. Cells containing plasmids conferring antibiotic resistance were grown in, or plated on media containing the appropriate antibiotic. Cells with plasmid conferring ampicillin resistance were grown in the presence of 0.1mg/ml ampicillin.

Section 2:10 Bacterial Strains

<u>Strain</u>	<u>References</u>
Y1090	Sambrook <u>et al.</u> , (1989)
TG2	Sambrook <u>et al.</u> , (1989)
TG1	Sambrook <u>et al.</u> , (1989)

Section 2:11 Preparation Of Competent Cells

E.coli cells were grown in a 10ml culture of L-Broth at 37°C with agitation to an O.D.₆₀₀ of 0.5. At this density the exponentially growing cells were removed from incubation and placed on ice. Cells were centrifuged at 3000g for 10mins, the supernatant was removed and the pellet was resuspended in 5ml of ice cold 100mM CaCl₂. The suspension was placed on ice for 20mins and the cells repelleted as before. The pelleted cells from this centrifugation step were resuspended in 0.5ml of fresh ice cold

100mM CaCl_2 . The transformation efficiency of these competent cells increases for 24hrs when stored on ice.

Section 2:12 Transformation Of Competent Cells

0.1ml of competent cells were mixed with DNA and placed on ice for 45mins with intermittent mixing. The cells were heat-shocked at 42°C for 3mins and then diluted to 1ml with L-Broth. Cells were incubated for 1 hour at 37°C , and then pelleted by brief centrifugation in a bench top microfuge and then resuspended in 100 μl of L-Broth prior to plating on appropriate L-Agar plates for overnight incubation at 37°C .

Section 2:13 Use Of Restriction Endonucleases

Restriction enzymes with their appropriate buffers were used according to the manufacturer's instructions.

Section 2:14 Growth, Plating And Storage Of Bacteriophage Lambda

a. Preparation Of Plating Bacteria

A single clony of Y1090 was grown overnight at 37°C with agitation in L-Broth containing 0.2% maltose. The cells were pelleted by centrifugation at 4000xg for 10mins at room

temperature and resuspended in 0.01M MgSO_4 to a final concentration of 1.6×10^9 cells/ml.

b. Plating Of Bacteriophage Lambda

100 μ l of bacteriophage lambda stored in SM (100mM NaCl, 5mM MgCl_4 , 50mM Tris-Cl pH 7.5, 0.1% gelatin) + chloroform (see below) was mixed with 60 μ l of Y1090 from above and incubated at 37°C for 20mins. 3ml of molten L-Agar containing 50ug/ml ampicillin and 0.2% maltose added when the L-Agar had cooled to 47°C was added to the bacteriophage lambda / Y1090 mixture. This was poured immediately onto dried L-Agar plates containing 50 μ g/ml ampicillin and 0.2% maltose. These overlaid plates were allowed to set and then they were inverted and incubated overnight at 37°C.

c. Storage Of Bacteriophage Lambda

The clear plaques produced above were sucked up into a Pasteur pipette and transferred to 1ml of SM containing 1 drop of chloroform. The bacteriophage lambda particles were allowed to diffuse out of the agar into the SM for 2hrs at room temperature before being stored at 4°C.

Section 2:15 Screening Of Lambda gt11 cDNA Library Using A ^{32}P Labelled cDNA Probe

a. Transfer Of DNA To Filters And Denaturation Of DNA

10^4 pfu of phage was plated out on 8.5cm L-Agar plates with 60 μ l of Y1090. These plates were incubated overnight at 37°C. Nitrocellulose filters were placed on the surface of these overnight plates for 3mins, the orientation of the filters being marked. The filters were transferred DNA side up to 3M whatman soaked in one of the four solutions below for the times indicated:-

1. 1.5M NaCl, 0.5M NaOH for 10mins
2. 0.5M tris-Cl pH8.0, 1.5M NaCl for 10mins
3. 2xSSPE (0.36M NaCl, 20mM Na₂HPO₄, 2mM EDTA pH7.7)
1% SDS for 10mins
4. 2xSSPE for 10mins

After the DNA had been denatured and neutralised it was air dried before being baked for 2hrs at 80°C under vacuum.

b. Nick Translation Of cDNA Probe

0.5 μ g of cDNA fragment to be labelled was added to 2.5 μ l of 10x nick translation buffer (0.5M Tris HCl (pH7.5), 0.1M MgSO₄, 1mM dithiothreitol and 500 μ g/ml of bovine serum albumin) containing 20nmoles of dATP, dCTP and dTTP in a 25 μ l reaction mix. To this was added 5 μ l of aqueous (³²P)dGTP (10 μ Ci/ μ l) (Amersham) and the reaction mix was made up to 21.5 μ l with ice cold water. 2.5 μ l of DNAaseI (10ng/ml in 1x nick translation buffer) was added and the reaction mix was incubated at 16°C for 1hr, The reaction was stopped by the addition of 1 μ l of 0.5M EDTA

(pH8.0) and the unincorporated dNTPs were removed by centrifugation through a sephadex G50 column (Sambrook et al., (1989). The labelled cDNA was ethanol precipitated and resuspended in water.

c. Hybridization Protocol

The baked filters from above were pre-hybridised for 1hr at 65°C with agitation in pre-hybridisation buffer (6xSSC, 5xDendardts solution, 0.5% SDS, 20µg/ml of dentured salmon sperm DNA). After pre-hybridization, the filters were hybridised at 65°C, overnight with agitation in hybridisation buffer (6xSSC, 5xDendardt's solution (1% (w/v) BSA, 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone), 0.5% SDS, 20µg/ml of dentured salmon sperm DNA) + 50ng/ml of nick translated cDNA probe. The filters were washed after overnight hybridisation with 2xSSPE for 15mins, 2xSSPE + 0.1% SDS for 30mins and finally 0.1xSSPE for 10mins, all of these washes were done at 65°C. The filters were air dried before being autoradiographed.

Section 2:16 Isolation Of DNA From Bacteriophage Lambda

a. Plate Lysate Of Bacteriophage Lambda

10⁵pfu of bacteriophage lambda were plated out with 100ul of Y1090 and incubated at 37°C overnight. This gave confluent growth overnight. 5ml of SM was added to the plate and incubated at 4°C for 4hrs with intermittent shaking. The SM was then harvested and

the plate was washed with 1ml of SM. One drop of chloroform was added to the pooled SM samples and stored at 4°C. The titre of the stock was estimated.

b Bacteriophage Lambda DNA Isolation
(Yamamoto et al., 1970)

250ml of L-Broth was inoculated with 2.5ml of overnight Y1090 and grown at 37°C with agitation until O.D.₆₀₀ reached 0.5 units. At this point 10^{10} pfu of bacteriophage lambda were added and the culture was incubated for a further 4hrs. After this 4hrs incubation 10ml of chloroform were added and the incubation was continued for a further 10mins. This culture was treated with 1µg/µl of DNase I and RNase for 30mins at room temperature. Solid NaCl was added to the DNase I and RNase treated solution to a final concentration of 1M and mixed. This was then left to stand on ice for 1hr. After this 1hr incubation solid polyethylene glycol (PEG 6000) was added to a final concentration of 10%(w/v) and dissolved by stirring slowly with a magnetic stirrer at room temperature. When the PEG was dissolved it was left to stand on ice for 1hr. The bacteriophage particles were recovered by centrifugation at 11000g for 10mins at 4°C. The bacteriophage pellet was resuspended in 4ml of SM. This was mixed with an equal volume of chloroform, and then centrifuged at 3000g for 15mins at 4°C. The upper aqueous phase was removed and 0.5µg/µl of solid CsCl was added and mixed. This mixture was then layered onto a 1.45/1.50/1.70g/ml CsCl step gradient. This gradient was centrifuged at 22000rpm for 2hrs at 4°C in a Beckman SW28. The bacteriophage band was collected from the interface

between the 1.45/1.50g/ml CsCl layers. The CsCl was removed from the above bacteriophage particles by dialysis against 1000-fold volume of dialysis buffer (10mM NaCl, 50mM Tris-Cl (pH8.0), 10mM MgCl₂) for 1hr at room temperature, followed by dialysis against 1000-fold fresh dialysis buffer. 20mM EDTA, 0.5% SDS and 50ug/ml proteinase K was then added to the dialysed bacteriophage particles, this was incubated at 37°C for 1hr. The protease treated mixture was then phenol/chloroform extracted and the final aqueous phase from the chloroform extraction was dialysed overnight at 4°C against three changes of 1000-fold volume of T.E.(pH8.0).

Section 2:17 Mini Preparation Of Plasmid DNA using The Boiling Method

Holmes and Quigley, (1981)

10ml aliquots of L-Broth containing 0.1mg/ml ampicillin were inoculated with individual colonies selected from L-Amp plates of transformed E.coli. The cultures were grown overnight at 37°C and 200rpm and then harvested by centrifugation at 2000g at room temperature for 10mins. The pelleted cells were resuspended in 100µl of 25% w/v sucrose, 50mM Tris-Cl pH8.0. These resuspended cells were transferred to Eppendorf tubes and 25µl of 10mg/ml lysozyme (prepared fresh) and 700µl of M-STET (5% sucrose w/v, 50mM Tris-Cl pH8.0, 50mM EDTA, 5% Triton x-100 v/v) was added. This mixture was placed in a boiling water bath for precisely 60secs. The lysed bacterial cells were placed on ice to cool

before being centrifuged for 1hr in a microfuge. The supernatant was removed and incubated with 1ul of 10mg/ml RNaseA at 37°C for 30mins. This RNase treated sample was then phenol /chloroform extracted and the DNA was precipitated with isopropanol. The final dried DNA pellet was resuspended in 100µl of H₂O.

Section 2:18 Preparation Of Plasmid DNA For Transcription

Based on Promega Biotec Technical Bulletin N°9.

The strain carrying the plasmid was grown overnight in 10ml of L-Broth containing antibiotic selection (usually 10µl of 50µg/ml ampicillin) at 37°C with agitation. 250ml of L-Broth containing 250µl of 50µg/ml ampicillin were inoculated with 2.5ml of overnight culture and grown overnight at 37°C with agitation. The cells of the second overnight culture were pelleted by centrifugation at 2000g for 10mins at 4°C with the brake on. This pellet was resuspended in 4ml of freshly prepared buffer 1 (page 101) and placed on ice for 15mins. After this 15mins incubation 8ml of buffer 2 (page 101) was added and mixed until the solution became transparent, this was then placed on ice for 10mins. After this 10mins incubation 5ml of 3M Na acetate pH4.6 was added and the solution was mixed until a white precipitate appeared, this was then placed on ice for 15mins. After this 15mins incubation the precipitate was pelleted by centrifugation at 17000g for 15mins at 4°C with the brake on. The supernatant was removed and 50µl of 1mg/ml RNaseA was added to the supernatant and incubated at 37°C for 30mins. The RNase treated supernatant was mixed with

0.5 volumes of phenol/chloroform (1:1) and left to stand for 15mins at room temperature. The mixture was centrifuged at 3000g for 15mins at room temperature. The upper aqueous phase was removed and mixed with 0.6 volumes of isopropanol and placed on ice for 15mins. The precipitated DNA was pelleted by centrifugation at 17000g for 10mins at 4°C. The precipitated DNA was resuspended in 1.6ml of H₂O, 0.4ml of 4M NaCl and 2ml of 13% PEG-6000. This mixture was placed on ice for 1hr. The PEG precipitated DNA was pelleted by high speed centrifugation in the bench top microfuge for 10mins at room temperature. The supernatant was removed using a drawn out Pasteur pipette. The remaining DNA pellet was washed once in 70% ethanol. The DNA pellet was dried under vacuum and then was resuspended in 50ul of H₂O.

Buffer 1: 25mM Tris-Cl pH8.0
 10mM EDTA
 15% Sucrose
 2mg/ml Lysozyme
 9ml H₂O
Buffer 2: 0.2M NaOH
 1% SDS

Section 2:19 Sequencing Using M13 Vectors

DNA was sequenced using a method based on the dideoxy chain termination method of Sanger et al., (1977). The single-stranded DNA template required by the above method was generated by

subcloning DNA into the single-stranded bacteriophage M13 (vectors mp18 or mp19). Bacteriophage particles containing single-stranded DNA are released into the growth medium during the replication of bacteriophage M13 DNA in infected bacteria. These particles can be rapidly isolated and single-stranded DNA purified for sequencing or mutagenesis.

a. Growth And Maintenance Of M13 Bacteriophage

DNA inserted into M13 bacteriophage vectors is unstable so incubations are kept below 5hrs and vectors were always stored long term as DNA not bacteriophage. Phage particles were regenerated from DNA by its transformation of competent TG1 or TG2 cells and plating out on H-plates in 3ml of melted H-top at 47°C after the addition of 100µl of TG2 cells, 40µl of 20mg/ml of 5-bromo-4-chloro-3-indolylβ-D-galactoside (x-gal), 40µl of 20mg/ml of isopropylthio-β-D-galactoside (IPTG). These plates were allowed to set before being inverted and incubated at 37°C overnight. Plaques were picked using a drawn out Pasteur pipette. Both of the M13 bacteriophage vectors used, mp18 and mp19 (Messing *et al.*, 1977) contain a polylinker region, they also contain regulatory sequences and the coding information for the first 146 amino acids of the beta-galactosidase gene (lac Z). This section of beta-galactosidase will complement host cells that contain a defective beta-galactosidase gene, that codes for an enzymatically inactive polypeptide lacking amino acids 11-14. Unsubstituted vectors plated on hosts carrying the defective beta-galactosidase gene will form blue plaques when the medium

contains IPTG and x-gal. Insertion of foreign DNA into the polylinker region of these vectors usually eliminates the above complementation resulting in clear plaques.

b. Preparation Of Single-stranded Template For Sequencing

10ml of 2xTY was inoculated with 0.1ml of an overnight culture of TG1 cells and a single recombinant plaque of bacteriophage M13mp18 containing DNA to be sequenced. This was then incubated for 5hrs at 37°C and 300rpm. 1ml of this 5hrs culture was transferred to an Eppendorf tube and centrifuged for 5mins in a microfuge. The supernatant was transferred to a fresh tube and mixed with 200µl of 20% polyethylene glycol 6000 (PEG) in 2.5M NaCl and left to stand for 15mins at room temperature. The PEG precipitate was pelleted by centrifugation in a microfuge for 5mins. The supernatant was discarded and the tube recentrifuged in a microfuge for 2mins and any remaining supernatant was removed with a drawn-out Pasteur pipette. The pellet was resuspended in 100µl of H₂O and 5µl of 10mg/ml RNaseA was added. This mixture was incubated at 37°C for 30mins. The RNase treated sample was then phenol / chloroform extracted and ethanol precipitated, the pelleted DNA was finally resuspended in 30µl of H₂O.

c. Sequencing Reaction Using Sequenase Enzyme

The sequenase enzyme was used to replace Klenow enzyme in the dideoxy chain termination sequencing reaction. The sequenase

enzyme provides high processivity, low 3' to 5' exonuclease activity, and the efficient use of nucleotide analogs over the Klenow enzyme.

1 μ g of single-stranded DNA template was annealed with 1 μ l of universal primer (0.5 μ mol/ μ l) in a total volume of 10 μ l of annealing buffer (40mM Tris-Cl pH7.0, 20mM MgCl₂, 50mM NaCl) by heating to 65°C for 2mins and then allowing to cool to below 35°C over a 30mins period. The sequencing reaction involves two steps, the labelling reaction and the termination reaction. To the annealed template primer reaction 1 μ l of 0.1M DTT, 2 μ l of labelling mix (1.5 μ M dGTP, 1.5 μ M dCTP, 1.5 μ M dTTP), 0.5 μ l of [α -³⁵S]dATP (10 μ Ci/ μ l Amersham) and 2 μ l of sequenase enzyme (1.6 units/ μ l) was added and incubated at room temperature for 5mins. After the labelling reaction 3.5 μ l of labelled template-primer reaction was added to Eppendorf tubes containing 2.5 μ l of ddATP (80 μ M dGTP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP, 8 μ M ddATP, 50mM NaCl), ddTTP (80 μ M dGTP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP, 8 μ M ddTTP, 50mM NaCl), ddCTP (80 μ M dGTP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP, 8 μ M ddCTP, 50mM NaCl), ddGTP (80 μ M dGTP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP, 8 μ M ddGTP, 50mM NaCl) termination mixes respectively. These were then incubated at 37°C for 10mins. The termination reaction reaction was stopped by the addition of stop solution (95% Formamide, 20mM EDTA, 0.05% Bromophenol Blue, 0.05% xylene Cyanol FF). The samples were stored at -20°C, and heated to 75°C for 2mins before being loaded on a 6% sequencing gel.

d. Preparation And Electrophoresis Of A Sequencing Gel

Samples from the sequencing reaction mixes were electrophoresed on 20x40x0.04cm slab gels for 1½hrs at 1500 constant volts. The electrophoresis gel plates, spacers and comb were cleaned carefully and allowed to dry before assembly. A 6% polyacrylamide 0.3% bisacrylamide, 700mM urea in 1xTBE gel was poured after the addition of TEMED and APS and allowed to polymerise for 1hrs. A slot was formed in the gel to accept the "sharks tooth" comb (0.4mm tick, 5.7mm point to point, BRL) prior to polymerisation. Once polymerised the gel was assembled onto the electrophoresis tank (BRL) The slot made to accept the comb was rinsed to remove any unpolymerised acrylamide or accumulated urea, and the comb placed in position. To warm the gel, and maintain a denaturing environment the gel was pre-run at 1500 constant volts for 20mins in TBE. Finally the wells formed by the comb were rinsed and the samples loaded.

Section 2:20 Oligonucleotide-directed in vitro mutagenesis

An Amersham kit was used for oligonucleotide-directed in vitro mutagenesis, this kit gave 80-90% generation of mutant over non-mutant plaques. These high efficiencies were achieved because of the removal of contaminating single stranded DNA by a filtration step, and the incorporation of thionucleotides in the generation of the mutatan strand such that it is resistant to

NciI digestion. The non-mutant strand may then be digested by NciI and removed by exonuclease III digestion.

a. Synthesis Of Mutagenic Oligonucleotides

The non-phosphorylated oligonucleotides were synthesised using an Applied Biosystems 380B DNA synthesiser and purified by high pressure liquid chromatography (HPLC).

b. Oligonucleotide Phosphorylation

The purified oligonucleotide was diluted to 20nmol/ml in H₂O. 2.5µl of this diluted oligo was mixed with 3µl of 10xkinase buffer (1M Tris pH8.0, 100mMMgCl₂, 70mM dithiothreitol and 10mM ATP) and 2 units of T4 polynucleotide kinase. The volume was made up to 25µl with H₂O before this mixture was incubated at 37°C for 15mins. After this incubation the kinase was inactivated by heating to 70°C for 10mins.

c. The Generation Of The Single-stranded Template To Be Mutagenised

The 150bp KpnI and PstI digested DNA fragment of p33K-2 was isolated by electroelution from a polyacrylamide gel (section 2:4). This was then ligated (section 2:8) into the sequencing vector M13mp18 which had been digested with KpnI and PstI and isolated from a LMP agarose gel (section 2:5) to generate M13mp18-33-150. The construction of M13mp18-33-150 was confirmed by sequencing analysis.

Single-stranded DNA was isolated by the inoculation of 10ml of 2xTY with 100µl of TG1 grown overnight and a recombinant

plaque. This was incubated for 4 hours at 37°C and 300rpm. After this incubation the phage infected cells were centrifuged in a microfuge to pellet the cells and the phage containing supernatant was stored overnight at 4°C. 100ml of 2xTY medium was inoculated with 1ml of an overnight TG1 and 1ml of phage supernatant as isolated above. This was incubated at 37°C and 250rpm for 4hrs. The supernatant was mixed with 0.2volumes of 20% (w/v) of polyethylene glycol (PEG 6000) in 2.5MNaCl and left to stand for 1hour on ice. The PEG precipitate was pelleted by centrifugation at 5000g for 20mins at 4°C. All traces of PEG were removed using a drawn-out Pasteur pipette. The PEG pellet was resuspended in 500ul of TE buffer. This was centrifuged in a microfuge to remove any remaining cells. The supernatant was mixed with 200ul of 20% (w/v) PEG 6000 in 2.5MNaCl and left to stand at room temperature for 15mins. This second PEG precipitate was pelleted by centrifugation in a microfuge for 5mins. The supernatant was discarded and the pellet recentrifuged and any remaining PEG was removed with a drawn-out Pasteur pipette. The viral pellet was resuspended in 500ul of TE buffer and phenol / chloroform extracted and ethanol precipitated. The ethanol precipitated pellet was washed in cold (-20°C) ethanol before being dried and resuspended in H₂O to a final DNA concentration of 1µg/ul.

d. Mutagenesis Reaction

5ul of single-stranded DNA template isolated above was mixed with 2.5ul of 1.6pmol/ul phosphorylated mutant oligonucleotide, 3.5ul of buffer 1 (Amersham) and 6ul of water. This mixture was

incubated at 70°C for 30mins and then placed at 37°C for 30mins so allowing the mutant oligonucleotide to anneal to the single-stranded template. The mutant DNA strand was synthesised and ligated by the addition of 5µl of MgCl₂ solution (Amersham), 19µl of nucleotide mix 1 (Amersham), 6µl of water and 6units of DNA polymerase (cloned Klenow fragment) and T4 DNA ligase respectively. This mixture was incubated at 16°C overnight. Any contaminating single-stranded DNA left after this overnight synthesis and ligation was removed by the addition of 30µl of 5MNaCl and 170µl of water. This was mixed and centrifuged through an Amersham filter unit at 500g for 10mins at room temperature. This filter unit consists of two nitrocellulose membranes that bind single-stranded DNA but allow the passage of double stranded DNA. The filter was washed with 100µl of 500mM NaCl and recentrifuged for 10mins. The DNA eluted from these two centrifugations was precipitated with 700µl of ethanol and 28µl of 3M sodium acetate. The pellet was resuspended in 25µl of buffer 2 (Amersham). 10µl of this resuspended pellet was digested with 5units of Nci1 and 65ul of buffer 3 (Amersham) for 90mins at 37°C. Only the non-mutant strand was digested by Nci1 as the mutant strand contains thionucleotides and so is not recognised by Nci1. The non-mutant strand was removed by digestion with exonuclease III. After the Nci1 incubation for 90mins 12µl of 500mMNaCl, 10µl of buffer 4 (Amersham) and 50 units of exonuclease III were added and this mixture was incubated at 37°C for 30mins. The exonuclease III was inactivated by heating the mixture to 70°C for 15mins. The mutant strand was repolymerised and ligated by the addition of 13µl of nucleotide mix 2

(Amersham), 5 μ l of MgCl₂ solution (Amersham), 3units of DNA polymerase 1 and 2units of T4DNA ligase. This mixture was incubated at 16°C for 3hrs. After this incubation 10 μ l of the above mixture was transformed into competent TG1 cells and plated out on H-plates (0.8% (w/v) bactotryptone, 100mM NaCl, 2% (w/v) bactroagar). These plates were incubated overnight at 37°C. Single-stranded DNA from three of the resultant plaques was isolated and sequenced to confirm mutation had taken place (section 2:19).

e. The reconstruction of the cDNA encoding the mutant pre-33K

The phage supernatant from the single stranded DNA preparation required for sequencing was used to infect 250ml of 2xTY already containing 2.5ml of overnight TG2. This culture was then grown at 37°C for 5 hours with agitation. Double stranded DNA was isolated from this culture as in section 2:17. The resulting double stranded DNA was digested with Kpn1 and Pst1 to release a 150bp fragment which was isolated from a 10% polyacrylamide gel by electroelution as in section 2:4. The transcription vector p33K-2* which contains the entire coding region for the 33K precursor but lacks the Kpn1 and Pst1 sites in the multiple cloning site (section 3:5:a) was also digested with Kpn1 and Pst1 to release a linear vector lacking the 150bp Kpn1 / Pst1 fragment. This linear vector was isolated from a LMP agarose gel (section 2:5). The 150bp insert containing the mutation was then ligated into the Kpn1 / Pst1 linearised p33K-2* vector (section 2:8) and used to transform competent TG2 cells and

plated out on L amp plates and incubated overnight at 37°C. Colonies from these plates were picked and plasmid DNA isolated for transcription.

f. Confirmation of above ligation

In the above ligation the vector control gave no colonies, so any colonies resulting in the ligation must contain the Kpn1 / Pst1 insert with mutation. To confirm this was the case the plasmid DNA isolated was digested with Kpn1 and Pst1 to release the 150bp insert. This insert was then ligated into M13mp18 which had previously been linearised with Kpn1 and Pst1 and isolated on a LMP agarose gel. The ligation mix was used to transform competent TG2 cells and plated out, the plates were incubated at 37°C overnight. A single plaque was picked and single stranded DNA was isolated from this. The resulting single stranded DNA was sequenced to confirm mutation as in section 2:19.

Section 2:21 Sodium Dodecyl Sulphate-Polyacrylamide Gel
Electrophoresis (SDS-PAGE)

(Laemmli, U.K., 1970)

a. Mini gel system

Two clean glass plates (one notched to receive a gel comb) were combined with suitable plastic spacers to form a mould for a polyacrylamide slab gel. The complete mould was placed into a plastic trough containing melted 1% (w/v) Agarose (sigma) which

once set acted as a plug.

A 15% (w/v) polyacrylamide gel solution was prepared according to the recipe below:

Resolving gel

		Final Concentration
Acrylamide/Bis-acrylamide (30%/0.8% (w/v))	10ml	15%/0.4%
3M Tris-Cl pH 8.8	2.5ml	375mM
H ₂ O	7.25ml	-
10% (w/v) SDS	0.2ml	0.10%
10% (w/v) ammonium persulphate	83μl	0.04%
TEMED	8.3μl	-

This solution was poured into the frame up to a level just below the position of the well forming comb. The resolving gel was overlaid with water saturated isobutanol and once set was removed by rinsing with H₂O and a stacking gel solution poured into the remaining space. A clean plastic well-former was inserted and the gel allowed to polymerise around it.

Stacking gel recipe

		Final Concentration
Acrylamide/Bis-acrylamide (30%/0.8% (w/v))	2ml	6%/0.16%
0.5M Tris-Cl pH 6.8	2.5ml	125mM

H ₂ O	5.0ml	-
10% (w/v) SDS	0.1ml	0.10%
10% (w/v) ammonium persulphate	50µl	0.05%
TEMED	7.5µl	-

After complete polymerisation the comb was gently removed and any unpolymerised acrylamide solution was washed from the wells. The gel was removed from the plastic trough, taking care to ensure the agarose plug remained in position and the gel was assembled into a gel tank. 500ml of 1x running buffer (25mM Tris-Cl pH8.3, 192mM glycine, 0.1% (w/v) SDS) was required to fill both upper and lower tank reservoirs. Samples for electrophoresis were prepared by diluting with an equal volume of 2x sample buffer, boiling for 2-3 minutes, and then loaded onto the gel. Electrophoresis was carried out at 35mM constant current for 2-2½ hours.

<u>Gel sample buffer (2x)</u>		<u>Final concentration</u>
0.5M Tris-Cl pH6.8	2.5ml	125mM
H ₂ O	0.5ml	-
Glycerol	2.0ml	20% (v/v)
10% (w/v) SDS	4.0ml	0.4% (w/v)
2-mercaptoethanol	1.0ml	10% (v/v)
Bromophenol blue	a few grains to colour	

b. Coomassie Blue Staining

Stain solution

0.5% (w/v) Coomassie Blue (Sigma) in 10% acetic acid
45% methanol

Immediately after electrophoresis gels were removed into the stain solution for 15-30 minutes with shaking.

Following staining the gels were removed into a destain solution of 10% Acetic acid and 45% methanol. Gels were destained until the background became clear.

c. Drying Of SDS-PAGE Gels

Polyacrylamide gels were dried onto 3 MM filter paper using a vacuum drier. A piece of 3 MM paper cut to size of the gels was placed in position on the drier. The paper was soaked with water, and the gel placed upon the paper. Cling-film was placed over the gel, and a piece of card laid on top to prevent the gel from cracking. With the gel in position, vacuum and heat were applied for one hour. The dried gel was removed and stored flat.

d. Fluorography and Autoradiography

Radioactively labelled proteins were visualised by fluorography and autoradiography.

Gels containing radiolabelled proteins were washed extensively in destain solution (10% acetic acid, 45% methanol) to (a) fix the proteins in the gels and (b) flush out any excess label not incorporated into the protein. Once fixed, the gel was placed in "Amplify" (Amersham) for 30 minutes and then dried as described in section 2:21:c. Finally, the dried gel was placed

into a light-proof cassette and exposed to X-ray film (Fuji),

Films were developed under dark-room conditions using LX24 developer (Kodak), and fixed with Unifix (Kodak), both diluted 1:4 with H₂O.

Section 2:22 Isolation Of Intact Chloroplasts For The Uptake Of Proteins

a. Preparation Of Purified, Intact Chloroplasts

Pea seeds Pisum sativum var. Feltham First (Booker Seeds, Nottingham) were grown in seed compost (Fisons) for 10 days under a 12 hour photoperiod. Light intensity was approximately 50uE/m²/sec and the temperature of the growth room was maintained at 20°C + or - 2°. The seeds were watered daily. After the 10 days of growth 100g of leaves were harvested and homogenised with a Polytron (Northern Media Supplies, Hull) at setting 8 for three 4 second bursts in a total volume of 400ml of partially frozen sucrose isolation medium; 0.35M sucrose, 2mM EDTA, 25mM HEPES-NaOH, pH7.6 (SIM). The leaf homogenate was filtered through 8 layers of muslin and the filtrate was centrifuged at 3000g for 2mins at 4°C. The pellets were resuspended in a small volume of ice-cold SIM using a cotton bud, pooled, and recentrifuged. The resulting pellet of washed chloroplasts was resuspended in 4ml of ice-cold sucrose resuspension medium 50mM HEPES-KOH pH8.0 and 330mM sorbitol (SRM).

Two 10ml Percoll step (80% / 62.5% / 45% / 27.5% / 10%) gradients were prepared as below:

	10%	80%
Percoll	1ml	8ml
H ₂ O	7ml	-
5xSRM	2ml	2ml

Percoll Solutions	27.5%	45%	62.5%
10%	3ml	2ml	1ml
80%	1ml	2ml	3ml

2ml of SRM resuspended washed chloroplasts were layered on top of each of the Percoll step gradients. The gradients were centrifuged at 1500g for 15 mins at 4°C. The intact chloroplast band was located at the 62.5% / 45% interface, clumped chloroplasts being at the 80% / 62.5% interface and broken chloroplasts at the 45% / 27.5% interface. The intact chloroplasts were removed using a cut off blue tip, diluted in SRM and centrifuged at 3200g for 2min at 4°C. The chloroplast pellet was washed once more with SRM and finally resuspended in SRM to give a chlorophyll concentration of 1mg/ml. Chlorophyll was determined according to Arnon (1949). Chloroplasts prepared in this way were used in chloroplast uptake studies.

b. The Uptake Of Precursors Into Isolated Chloroplasts

Uptake of precursors into isolated chloroplasts was studied using an assay procedure modified from that of Chua and Schmidt, (1978). Uptake incubation mixture contained 10µl of in vitro wheat germ translation, 10ul of 200mM D,L-methionine, 7µl of

100mM ATP in SRM, 3.5 μ l of 200mM MgCl₂ in SRM, 3 μ l of 5xSRM and 55 μ l of intact chloroplasts at 1mg/ml chlorophyll concentration. Mixtures were incubated under illumination (100 μ E m⁻²sec⁻¹) at 25°C with gentle agitation. After the required incubation, usually 20 minutes, the mixture was divided in half and one half was left on ice for 40 minutes. To the other half 2.5 μ l of trypsin (5mg/ml in SRM (Sigma)) was added and the sample was left on ice for 40 minutes. After the 40 minute incubation the chloroplasts were washed in 10ml of ice cold SRM and pelleted by centrifugation at 3200g for 2 minutes at 4°C. The pellets were resuspended in 60 μ l of 10mM Tris pH7.6 containing 100 μ g of soybean trypsin inhibitor and then mixed with one volume of 2x sample buffer, and boiled for 2 minutes before analysis by SDS-polyacrylamide gel electrophoresis followed by fluorography.

c. The Localization Of Imported Precursor To The Thylakoid Lumen

The above uptake experiment localises the precursor to a protease protected position within the chloroplast, it is also possible to localise thylakoid lumen precursors to a protease protected position within the thylakoid lumen. For this the uptake mixture was quadruple. After uptake in the light a quarter of the mixture was washed in ice cold SRM and pelleted at 3200g for 2 minutes at 4°C before being resuspended in 60 μ l of 10mM Tris pH7.6 and one volume of 2x sample buffer and boiled for 2 minutes. The rest of the uptake mixture was treated with 7.5 μ l of trypsin (5mg/ml in SRM) for 40mins on ice. This was then washed

in 10ml of ice cold SRM and pelleted by centrifugation at 3200g for 2 minutes at 4°C. The pellet was resuspended in a total volume of 180ul of 10mM Tris pH7.6 containing 300µg of soybean trypsin inhibitor (Sigma). 60µl of this resuspended chloroplast pellet was added to one volume of 2x sample buffer and boiled for 2 minutes. The rest of the resuspended chloroplast pellet was transferred to a 1.5ml microfuge tube and centrifuged in a microfuge for 10mins at 4°C. The supernatant was added to one volume of 2x sample buffer and boiled for 2 minutes. The pelleted chloroplast were lysed in a total volume of 120µl of ice cold 10mM Tris-Cl pH7.6. 60µl of this resuspended pellet was added to one volume of 2x sample buffer and boiled for 2 minutes. 8µl of proteinase K (1mg/ml in SRM Sigma) was added to the other 60µl and left for 30 minutes on ice. After the 30 minutes incubation the thylakoid fraction was washed twice in 1ml of ice cold 10mM Tris-CL pH7.6, the thylakoids being pelleted by centrifugation in a microcentrifuge. The pellet was finally resuspended in 60µl of 10mM Tris pH7.6 and one volume of 2x sample buffer and boiled for 2 minutes. All these samples were analysed by SDS-polyacrylamide electrophoresis followed by fluorography.

Section 2:23 In vitro Transcription Of p33K-2

(Krieg and Melton, 1986)

The full length cDNA for the pre-33K of wheat was sub-cloned into the EcoR1 site of the transcription / translation vector pGEM-4Z to create p33K-2 (figure 10). DNA was isolated by the large scale method (section 2:18) and adjusted to a concentration

of 1µg/µl.

2µl of (1µg/µl) linearised DNA was incubated with 15.5µl transcription premix (40mM Tris-Cl pH7.5, 6mM MgCl₂, 2mM Spermidine, 10mM DTT, 500uM rATP, rCTP, rUTP, 50µM rGTP (Promega nucleotides), 100µg/ml BSA (nuclease free BRL), 1 unit/µl ribonuclease inhibitor (Promega) plus 1µl of 15units/µl SP6 or T7 RNA polymerase (BRL). To produce capped transcripts (for increased translational activity and greater stability) 1µl of G(5')pp(5')G(monomethyl cap) was added to a final concentration of 500µM (Pharmacia). The reaction mix was incubated at 40°C for 30 minutes and then a further 30 minutes after the addition of 0.5µl 20mM GTP (final concentration 0.6mM). Reaction products were stored at -80°C.

Section 2:24 Translation Of RNA Transcripts In A Wheatgerm Lysate System

Wheatgerm lysate was prepared as described by Roberts & Paterson, (1973) and Anderson, (1983).

1µl of transcription reaction mix containing newly transcribed RNA was incubated at 27°C for 1 hour with 3.75µl wheatgerm lysate, 2.4µl translation premix, 1µl of [³⁵S] methionine 30 TBq/nmol (10µCi/µl) made up to 12.5µl with H₂O. The wheatgerm translation premix was composed of 20mM Hepes KOH pH7.6, 1mM ATP, 8mM creatine phosphate, 40µg/ml creatine phosphokinase 30µg/ml spermidine pH 7.0, 2mM DTT, 20uM GTP, 25uM amino acid mix (from 5mM stock excluding methionine (Anderson, 1983). Products from the wheatgerm lysate translation were

separated by SDS-PAGE and visualised by fluorography and autoradiography.

Section 2:25 In vitro Assays Of Precursor processing By Thylakoidal Processing Peptidase (TPP), Stromal Processing Peptidase (SPP) And Leader Peptidase (LEP)

Precursors for processing assays were generated using an in vitro wheat germ transcription / translation system. 1 μ l of precursor was either processed with 19 μ l of TPP (provided by Tricia Kirwin (Warwick)), 19 μ l of SPP (provided by Janet Musgrove (Warwick)), 19 μ l of a stromal fraction (prepared by lysing chloroplasts at 1mg/ml of chlorophyll in 10mM Tris-Cl pH8.0 and centrifugation in microfuge for 20mins at 4°C taking the supernatant as the stromal fraction) or 1 μ l of LEP (kindly provided by W. Wickner (UCLA)) and 18 μ l of 20mM Tris, 0.015% Triton-X100 for 1½ hours at 27°C. After processing the samples were mixed with one volume of 2xsample buffer and boiled for 2 minutes before analysis by SDS-polyacrylamide gel electrophoresis followed by fluorography.

Section 2:26 Uptake Of Precursors Into Isolated Thylakoids

Studies of the uptake of precursors into isolated thylakoids were based on the method of Mould et al., (in press). Intact chloroplasts were isolated as in section 2:22 and were washed twice in sucrose resuspension medium 50mM HEPES-KOH pH8.0 and

330mM sorbitol (SRM). The chloroplasts were pelleted in a microfuge for 5 minutes at 4°C. The final pellet was lysed in 10mM HEPES pH 8.0 and the chlorophyll concentration was determined according to Arnon, (1949) and adjusted to 1mg/ml. The chloroplasts were left on ice for 30 minutes and were then pelleted in a microfuge for 5 minutes at 4°C. The stromal supernatant was retained and the pellet was washed in a microfuge at 4°C and resuspended in SRM to a chlorophyll concentration of 1mg/ml. 25µl aliquots of this thylakoid suspension were pelleted in a microfuge for 5 minutes at 4°C before being resuspended in 30µl of stroma and 5µl of 75mM MgCl₂ and 5µl of precursor. This uptake mixture was incubated under illumination (100µE m⁻²sec⁻¹) at 25°C with gentle agitation for 25 minutes. After uptake incubation, half of the mixture was added to one volume of 2xsample buffer, and boiled for 2 minutes. The other half was mixed with 2µl of 1mg/ml of proteinase K (made up in SRM) for 30 minutes on ice. The proteinase K was inhibited at the end of the 30 minute incubation by the addition of 50µl of 7mg/ml PMSF (phenylmethanesulphonyl fluoride)(made up in ethanol). The thylakoids were then washed in 1ml of 10mM Tris pH7.6 and pelleted in a microfuge at 4°C for 10 minutes. The protease treated thylakoid pellet was resuspended in 18.5µl of 10mM Tris-Cl pH 7.6 and 1.5µl of 7mg/ml PMSF (made up in ethanol) before being added to one volume of 2xsample buffer and boiled for 2 minutes. All samples were analysed by SDS-gel electrophoresis and fluorography.

Section 2:27 Western Blotting Biotin-streptavidin / 4 Chloro-1-Napthol Method

Proteins initially separated by SDS-PAGE were transferred onto nitrocellulose paper using the Ancos (Denmark) semi-dry transblotting apparatus. Transfer was achieved by sandwiching the polyacrylamide gel next to the nitrocellulose paper such that when an electrical current was applied, proteins would migrate from the gel and bind in the same relative positions upon the nitrocellulose paper the transfer buffer was [$\frac{1}{4}$ laemmli, 20% (v/v) methanol] and blotting was carried out at 400 volts for 2hrs.

Following blotting the gel and nitrocellulose sandwich were separated. The gel was coomassie blue stained (section 2:21:b), whilst the nitrocellulose filter was placed into PBS containing 2% "Marvel" milk product. Three 10 minute washes in the "Marvel" solution were adequate to prevent non-specific binding of antibodies to the filter. The filter was then probed with specific antibodies (a polyclonal antibody provided by Janet Musgrove (Warwick) to 33K of pea)) in 5ml Of "Marvel" solution overnight at room temperature. Following incubation the filter was washed three times in PBS containing 0.1% Tween and then incubated in 5ml of PBS containing a 1:300 dilution of biotinylated-protein A (Amersham) for 1 hour at 30°C. The filter was then washed in PBS containing 0.1% Tween and further incubated with 5ml of PBS containing a 1:300 dilution of Streptavidin-horseradish peroxidase complex (Amersham) for 30 minutes at 30°C. The filter was washed in PBS containing 0.1% Tween once followed by a wash in just PBS prior to developing.

The filter was developed after the final PBS wash by mixing together solutions containing 0.5mM Tris-Cl pH7.4, 125mM NaCl in a final volume of 50ml, and 30mg of 4 chloro-1-Naphthol dissolved in 10ml of methanol together with 20 μ l of 20% (v/v) hydrogen peroxide. The filter was then washed in the mixture of these two solutions for approximately 30 minutes. The reaction was stopped by washing in water and air drying the filters.

Section 2:28 Escherichia coli Expression Of Mutants

Pre-33K cDNA was subcloned into the E.coli expression vector pKK233-2 to create pKK233-2-33. When expression of pKK233-2-33 was induced with 1mM IPTG a mature sized protein was seen in the periplasmic space (Meadows et al., in press). It would appear from the above expression of pre-33K cDNA that the E.coli export machinery can recognise the pre-sequence of 33K precursor and so facilitate the transport of this precursor across the E.coli periplasmic membrane. LEP has previously been shown by Halpin et al., (1989) to cleave pre-33K to a mature sized protein. It was not therefore unexpected that pre-33K would be cleaved by LEP if it was exported across the periplasmic membrane of E.coli. For a review of the similarities between the thylakoid import and E.coli export machinery see section 1:11:b:ii.

The Pst1 / Kpn1 inserts containing the ten mutations created in section 2:20:d and isolated in section 2:20:e were subcloned into the pKK233-2-33 vector which had previously been linearised with Pst1 and Kpn1 and isolated on a low melting point agarose gel (section 2:5 to create pK233-2-33-2(-1Leu), etc.

The above mutant E.coli expression vectors were grown in 5ml of L-Broth containing 5µl of 50µg/ml of ampicillin overnight at 37°C with agitation. 50µl of this overnight culture was used to inoculate a fresh 5ml of L-Broth containing 5µl of 50µg/ul of ampicillin. This was grown at 37°C with agitation until the O.D.₆₀₀ reached 0.2 units. The expression vector was induced by the addition of IPTG to a final concentration of 1mM. This induced culture was grown for a further 3hrs at 37°C with agitation. After this incubation 1ml of the culture was pelleted by centrifugation in a microfuge for 10 minutes. The pelleted cells were resuspended in 50µl of L-Broth and added to a equal volume of 2x sample buffer and boiled for 2 minutes. The samples were analysed by SDS-PAGE (section 2:21) followed by Western blotting (section 2:27).

Section III RESULTS AND DISCUSSION

Section 3:1 Introduction

The first cDNA encoding a thylakoid luminal precursor to be isolated was that of Silene pratensis plastocyanin (Smeekens et al., 1985), it was shown to contain an N-terminal pre-sequence which was assumed to contain the targeting signals required for import into chloroplasts. An isolated chloroplast assay as well as partially purified extracts of SPP and TPP were used by Hageman et al., (1986) and Smeekens et al., (1986) to elucidate the mechanism of import across the chloroplast envelope and intra-organellar routing to the thylakoid lumen. These workers were able to show that the plastocyanin precursor (pre-PC) was processed by SPP to a polypeptide intermediate in size between the precursor and mature forms and this intermediate was further processed by TPP to a mature sized polypeptide. This work lead to the initial formulation of the two step model.

At the start of my project it was still unclear whether the import of pre-PC into chloroplasts represented a common import route for all thylakoid luminal proteins, or whether other routes of import were followed by other luminal proteins. The initial aim of my work was to isolate another cDNA encoding a thylakoid lumen targeted protein (pre-33K), and to use this protein to confirm or refute the two step model as a general model for the import of polypeptides into the chloroplast and their intra-organellar targeting to the thylakoid lumen.

The N-terminal pre-sequence of proteins such as rubisco SSU had been shown to contain the targeting signals required to cross

the chloroplast envelope (Van de Broeck *et al.*, 1985) and it was likely that the targeting signals of thylakoid lumenal polypeptides such as pre-PC and pre-33K would also be contained in their pre-sequences. The second objective of my work was to locate and characterise the targeting signals present in pre-33K. During the course of the isolation of the cDNA encoding pre-33K it was becoming clear that the N-terminal region (ETD) of the pre-sequence of thylakoid lumen polypeptides was required for targeting across the chloroplast envelope, and the C-terminal region (TTD) was necessary for import into the thylakoids. This was based on the targeting of foreign proteins into isolated chloroplasts using the PC and 33K pre-sequences (Meadows *et al.*, 1989; Hageman *et al.*, 1990), deletion analysis using the PC pre-sequence (Hageman *et al.*, 1990) and sequence comparisons with other pre-sequences published during this period (von Heijne *et al.*, 1989). A biochemical approach was adapted using a stromal extract and the chloroplast uptake assay to confirm the requirement for the ETD in the targeting of pre-33K and pre-23K across the chloroplast envelope (the clone encoding the pre-23K polypeptide was isolated shortly after the pre-33K clone by H. James (Warwick)). A similar approach using partially purified TPP and a thylakoid uptake assay was to be used to confirm the role of the TTD, but the efficiency of cleavage by TPP of pre-23K and pre-33K was poor, making the results difficult to interpret.

One of the aims of my work was to test the validity of certain aspects of the two step model, specifically with regard to the role of SPP in the import pathway. Although it is clear that lumenal precursors cloned to date (pre-33K, pre-23K and pre-PC) contain an SPP cleavage site, these data do not show that SPP

cleavage is an obligatory step in the import pathway. An aim in my project was to block cleavage by SPP and test the subsequent effect on the import pathway. The approach taken involved testing the effects of carboxymethylation of precursors. Since this procedure had previously been used by Robinson & Ellis, (1984) and Musgrove & Robinson, (in prep.) to completely block cleavage by SPP of two stromal protein precursors (those of SSU and acyl carrier protein (ACP)).

The more refined technique of site directed mutagenesis was also used to characterise the targeting signals present in the pre-sequence of pre-33K. Von Heijne et al., (1989) has shown that the pre-sequences of thylakoid lumen targeted proteins contain alanine at their -1 and -3 residues, with the exception of valine at the -3 residue in the 16kDa OEC polypeptide of spinach and serine at the -3 residue in the CF₀-II polypeptide of spinach. These residues have been implicated in the TPP cleavage event. After the sequence analysis of the 5' end of the pre-33K clone, site direct mutagenesis was used to substitute a variety of amino acids at the -1 and -3 positions of pre-33K in order to elucidate their role in the TPP cleavage mechanism and characterise the substrate requirements of TPP. The work of Folz et al., (1988) and Fikes et al., (1990) have helped define the sequence requirements of LEP and SP for their respective targeting signals, a comparison of the three peptidases sequence requirements may be informative about a common mechanism. The mutants generated were analysed using SPP, TPP and the isolated chloroplast import assay.

The results section will first consider the isolation of a cDNA for pre-33K and its characterisation as a chloroplast

targeted polypeptide. The function of the pre-sequence during import into chloroplasts is then characterised using:

1. Isolated peptidase and chloroplast import assays.
2. Chemical modification of cysteine residues.
3. Site directed mutagenesis of the -1 and -3 residues.

Section 3:2 Isolation Of A Full Length cDNA Encoding pre-33K

It was anticipated that a full length cDNA to the 33K OEC precursor (pre-33K) of wheat would be available at the start of this project; this, however, was not the case. Prior to the start of this project Dr. J.W.Meadows and I had isolated a partial cDNA encoding pre-33K by the screening of a wheat lambda gt11 expression library, using antibodies raised to the mature 33K. I and Dr. J.W. Meadows used this partial cDNA to screen a lambda gt11 library for a full length cDNA.

The expression library was constructed so that the cDNA fragments were inserted into the lambda gt11 vector downstream of the 3'end of the lacZ gene, resulting in the production of a fusion-protein (when in the correct reading frame) (Yang & Davies, 1983). Bacteria infected with phage producing this fusion protein can then be identified using specific antisera. The first antibody screening of the lambda gt11 library had isolated a cDNA which coded for a polypeptide of 200 residues, which showed 74% homology with a spinach 33K clone isolated by Tyagi et al., (1987).

The second screen (undertaken by myself and Dr. J.W.Meadows) of the lambda gt11 library (kindly provided by Dr.C.Raines (Essex)) used the partial cDNA isolated from the antisera screen

to probe the library. The partial cDNA was first labelled with P^{32} by nick translation and was then used as a hybridization probe to probe the library. The bacteria infected with phage producing DNA that anneals to the labelled cDNA probe could be identified by virtue of their ^{32}P label.

During the second screen, two plaque-pure clones were isolated from an initial screen of 10^5 plaques and lambda bacteriophage DNA was extracted (section 2:16); these clones contained 1.4 and 1.3kbp inserts respectively when the lambda bacteriophage DNA was digested with EcoR1. Both of these inserts were subcloned into the EcoR1 site of the transcription / translation vector pGEM-4Z (figure 10), to create p33K-1 (which contains the 1.4kbp insert) and p33K-2 (which contains the 1.3kbp insert). The transcription / translation vector pGEM-4Z contains a polylinker region bounded by the promoter regions for the SP6 and T7 RNA polymerase. Both p33K-1 and p33K-2 were transcribed using SP6 and T7 RNA polymerases (section 2:23) to generate RNA which was then used to programme an in vitro wheat germ translation system (section 2:24). Figure 11 shows the translation products of such an experiment separated by SDS-PAGE followed by fluorography. Only p33K-2 gave rise to a labelled product when RNA was generated, using SP6 polymerase (lane 2), the resulting translation product was sized at 42kDa.

Section 3:3 Characterisation of the translation product
 generated by p33K-2

In order to confirm that p33K-2 encodes pre-33K, the 5' end of the cDNA was sequenced and its deduced amino acid sequence was

Figure 10 The subcloning of the 1.3kbp and 1.4 kbp cDNAs into
the transcription / translation vector pGEM-4Z

The 1.3 and 1.4 kbp cDNAs were released from the lambda DNA by digestion with EcoR1, and the resulting DNA fragments were isolated from an agarose gel. The transcription /translation vector pGEM-4Z was linearised by digestion with EcoR1 and its 5' phosphate was removed to prevent vector religation. The linearised pGEM-4Z and cDNA fragments were ligated together to create p33K-1 (which contains the 1.4kbp insert) and p33K-2 (which contains the 1.3kbp insert).

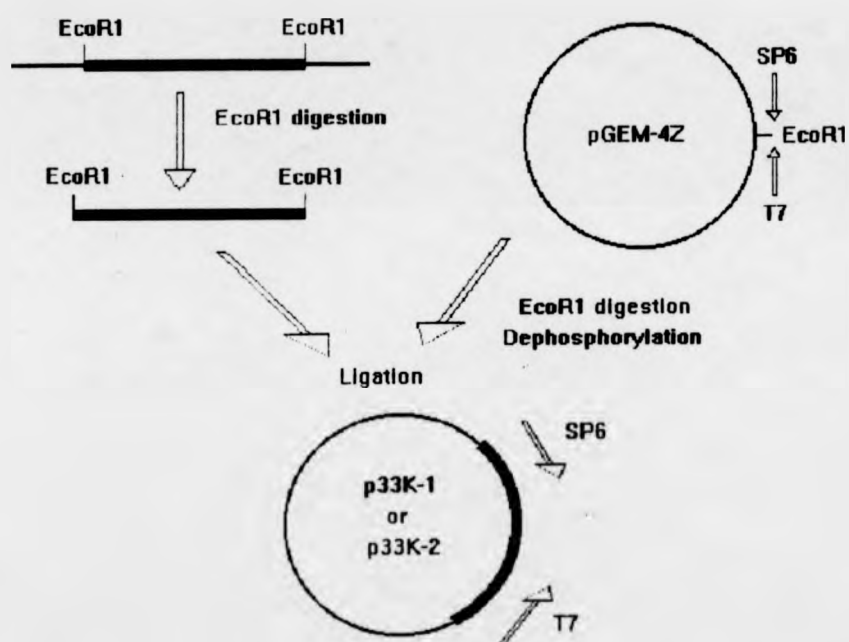
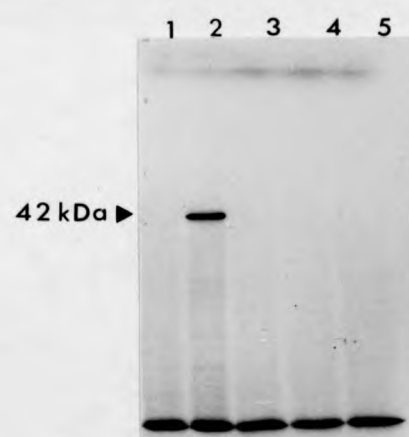


Figure 11 In vitro transcription and translation of p33K-1 and p33K-2

The vector p33K-2 was transcribed with SP6 (lane 2) and T7 (lane 3) RNA polymerases followed by translation of resultant RNA in a wheat germ system. The vector p33K-1 was also transcribed with SP6 (lane 4) and T7 (lane 5) RNA polymerases and the resulting RNA was translated in a wheat germ system. In lane 1 the RNA component of the translation system was replaced by water. After incubation the reactions were stopped by the addition of one volume of 2Xsample buffer and boiling for 2mins. Samples were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography.



compared with that of the spinach 33K clone (Tyagi et al., 1987)(section 3:4). Confirmation that the 42kDa translation product was a chloroplast targeted precursor came from in vitro processing studies using SPP and TPP (James et al., 1989) and isolated chloroplast import studies. Hageman et al., (1990) had previously used such studies to show that pre-PC was imported into chloroplasts via a stromal intermediate.

When the 42kDa translation product was incubated with isolated chloroplasts it was taken up and processed to a 33kDa protein (figure 12). This polypeptide was protected from externally added protease (lane 2) and was found in the thylakoid membrane fraction when the chloroplasts were lysed and centrifuged (lanes 3 and 4). The 33kDa protein was resistant to protease digestion of the thylakoids (lane 5) indicating a luminal location. These results provided firm evidence that p33K-2 encoded pre-33K; final proof was later obtained when the entire cDNA was sequenced (Meadows & Robinson, in press).

James et al., (1989) used partially purified extracts of SPP and TPP to process pre-33K to its stromal intermediate, int-33K (figure 13, lane 1) and to its mature form, 33K (lane 2). The processing pattern obtained using SPP and TPP, and the uptake into isolated chloroplasts and routing of pre-33K to the thylakoid lumen are all similar to those shown previously for pre-PC. Pre-33K processing and chloroplast import data confirms the initial observation of Hageman et al., (1984); Chia et al., (1986); Smeekens et al., (1986) that thylakoid lumen precursors are targeted via a stromal intermediate and suggest that this route may represent the import route used by the majority of luminal precursors.

Figure 12 The import of pre-33K into intact chloroplasts and its localization to the thylakoid lumen

Pre-33K translation mixture (lane T) was incubated with intact chloroplasts at 25°C for 20mins under illumination after which the chloroplasts were either washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol and then lysed in 10mM Tris pH8.0 (lane 1) or were washed and treated with 0.2mg/ml of trypsin for 40mins on ice. After incubation, these chloroplasts were washed with 50mM HEPES-KOH pH8.0, 330mM sorbitol, 0.2mg/ml of trypsin inhibitor, and either analysed directly (lane 2) or after lysis in 10mM Tris pH7.6 and fractionation into stromal (lane 3) and thylakoidal fractions. The thylakoidal fraction was either analysed directly (lane 4) or was treated with 0.05mg/ml proteinase K for 30mins on ice before being washed in 10mM Tris pH7.6, 1mg/ml PMSF and resuspended in 10mM Tris pH7.6. All samples were added to one volume of 2x sample buffer and boiled for 2mins, and were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation: pre-33K, int-33K and 33K are the precursor, intermediate and mature forms of the 33kDa OEC polypeptide.

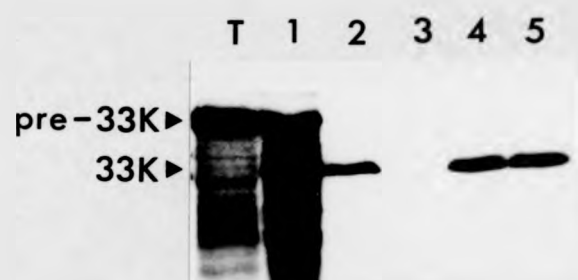
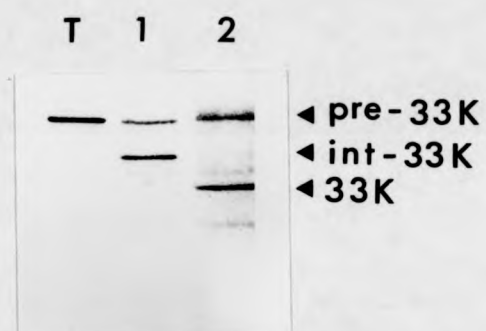


Figure 13 Processing of pre-33K by SPP and TPP

Pre-33K translation products (lane T) was incubated with SPP at 27°C for 90mins (lane 1) and with TPP at 27°C for 90mins (lane 2). After incubation the reactions were stopped by the addition of one volume of 2x sample buffer and boiling for 2mins. Samples were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as for figure 12. Figure taken from James et al., (1989).



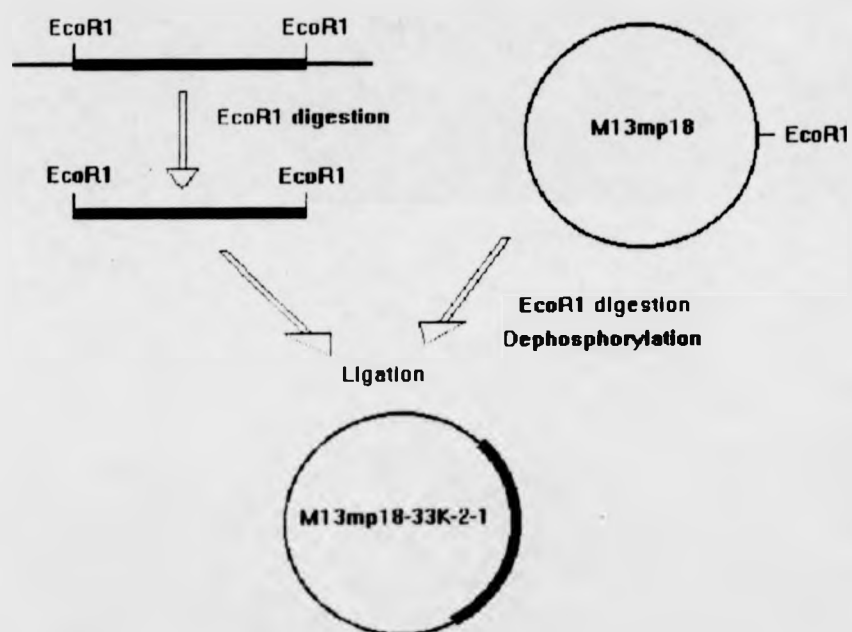
Section 3:4 Sequencing Of The Pre-sequence Of The Full Length
Pre-33K cDNA

The 42kDa translation product encoded by p33K-2 has been shown to be targeted to the thylakoid lumen via an intermediate. DNA sequence analysis of the cDNA and its comparison with the sequence of the spinach 33K clone (Tyagi et al., 1987) would allow the confirmation that the 42kDa translation product is in fact pre-33K. During the isolation of the 33K clone a number of other cDNAs encoding thylakoid luminal precursors were isolated and sequenced. Von Heijne et al., (1989) have compared these sequences and were able to identify a number of common features that may act as targeting signals. In order to characterise possible targeting signals in the 33K pre-sequence the 5' end of the cDNA was sequenced and the amino acid sequence deduced. The structure of the 33K pre-sequence would also be useful in the interpretation of the results of carboxymethylation of pre-33K. The DNA sequence around the terminal cleavage site was also required for the generation of the oligonucleotides used in the site directed mutagenesis of the -3 and -1 residues.

The first step in the sequencing of the 5' end of the 33K cDNA was to subclone the 1.3kbp EcoR1 fragment present in the lambda bacteriophage into the EcoR1 site of the DNA sequencing vector M13mp18 (figure 14). The orientations of the resultant ssDNA clones were determined with respect to the already sequenced partial cDNA of the pre-33K. The DNA sequencing method used allowed the reading of 250bp; within this first 250bp a unique Pst1 restriction enzyme site was located. A second sequencing vector was created which lacked the 5' end of the cDNA

Figure 14 The subcloning of the 1.3kbp cDNA into the sequencing vector M13mp18

The 1.3kbp cDNA was released from the lambda DNA by EcoR1 digestion, the resulting DNA fragment was isolated from an agarose gel. The sequencing vector M13mp18 was linearised by digestion with EcoR1 and the 5' phosphates were removed to prevent vector religation. The 1.3kbp DNA fragment was ligated into the linearised sequencing vector M13mp18 to create M13mp18-33K-2-1 when the DNA fragment was ligated in a 5'-3' orientation (ligated so that the transcriptional start site is proximal to the sequenceing priming site).



up to this PstI site, and a further 250bp were sequenced, enabling all of the sequence encoding the pre-sequence to be read. This initial sequencing was confirmed by DNA sequencing the complete 33K cDNA (performed by Dr. J. Meadows (Warwick)). Figure 15 shows the DNA sequence and deduced amino acid sequence from the initial sequencing of the full length cDNA and the PstI digested M13mp18-33K-2-1. The PstI and KpnI sites are shown as is the putative start ATG, and the SPP and the TPP cleavage sites. The SPP and TPP cleavage sites have recently been assigned by the protein sequencing of the intermediate and mature forms of 33K (D. Bassham and Dr. P. Elderfield (Warwick)) although I made an unsuccessful attempt to protein sequence the mature form of 33K (below).

The 33K pre-sequence shares a number of features that have previously been shown to be common to those of other nuclear-encoded thylakoid lumen proteins. These include the presence of small uncharged hydrophobic residues located at the -1 and -3 positions of the pre-sequence (usually alanine) relative to the terminal processing site, a conserved methionine and alanine residue at the start of the pre-sequence, and a group of hydrophobic residues located between -14 and -5. The ETD and TTD domains have been assigned on protein sequence data of the mature and intermediate polypeptides produced by cleavage by partially purified fraction of TPP and SPP (provided by Dr. P. Elderfield and D. Bassham (Warwick))(Figure 15).

An attempt was made by myself and Dr. B. Dunbar (Aberdeen) to protein sequence the int-33K. The int-33K was generated by the in vitro cleavage of ^3H Ala labelled pre-33K by a partially purified SPP extract. It was not clear if this attempt failed

Figure 15 The DNA sequence and the deduced amino acid sequence of the 33K pre-sequence

To sequence the 33K pre-sequence the 1.3kbp insert isolated by the EcoR1 digestion of lambda bacteriophage DNA was ligated into the EcoR1 site of the sequencing vector M13mp18 to create M13mp18-33K-2 figure 14, this ligation was used to transform competent TG1 cells (sections 2:11 and 2:12), and plated out onto H-plates (section 2:14). A number of plaques were picked and single stranded DNA was isolated (section 2:19); the orientation of the insert in these M13mp18-33K-2 clones was determined by their ability to anneal with the single stranded DNA of the already sequenced partial cDNA clone (section 2:19). The single stranded DNA of a 5' to 3' orientated clone (M13mp18-33K-2-1) was sequenced (section 2:19), analysis of this sequence data identified a Pst1 site located 167bp from the start of the clone. Restriction enzyme analysis of the replicative form of M13mp18-33K-2-1 confirmed that this was the only Pst1 site in the cDNA. The replicative form of M13mp18-33K-2-1 was digested with Pst1 to release an approximately 150bp insert (section 2:13); the Pst1 linearised M13mp18-33K-2-1 DNA was isolated from a LMP agarose gel (section 2:5), and re-ligated (section 2:8) before being used to transform competent TG2 cells (sections 2:11 and 2:12) and plated out onto H-plates (section 2:14). Single stranded DNA was isolated from the resultant plaques and sequenced (section 2:19).

The DNA sequence resulting from the sequencing of M13mp18-33K-2-1 the Pst1 digested M13mp18-33K-2-1 clone is shown; the deduced amino acid sequence is also shown. The TPP and SPP

processing sites are indicated as are the deduced envelope and thylakoidal transfer domains (the protein sequence data required for the assignment of the TPP and SPP processing sites was kindly provided by D.Bassham and Dr.P. Elderfield(Warwick)). The unique PstI and KpnI sites are underlined. A number of features common to other nuclear encoded thylakoid lumen targeted proteins are also shown: the conserved Met, Ala at the start of the pre-sequence, the hydrophobic domain in the TTD, and small hydrophobic residues located at the -3 and -1 positions. The numbering refers to the amino acid residues. The cysteine residues are indicated by an asterisk.

CTC CCG CGT ACC AAG CCA CCG ACC AAC ATG GCA GCG TCT CTC CAA GCC GCG GCC ACC GCG CTG ATG CCG GCC AAG ATC
 Met Ala Ala Ser Leu Gln Ala Ala Thr Leu Met Pro Ala Lys Ile

 Met Ala

-60
 GGC GGC CCG GCC TCC TCC GCG CGA CCG TCG CAC GTC GCC GCC CGG GCG TTC GGC GTC GAC GCT GGC GCC AGG ATC
 Gly Gly Arg Ala Ser Ser Ala Arg Pro Ser Ser His Val Ala Arg Ala Phe Gly Val Asp Ala Gly Ala Arg Ile

 Envelope Transfer Domain-----
 SPP

*
 ACC TGC TCC CTG CAG TCC GAC ATC AAG GAG GTC GCA AGC AAG TGC GCC GAC GCC AAG ATG GCC GCG TTC GCC
 Thr Cys Ser Leu Gln Ser Asp Ile Arg Gln Val Ala Ser Lys Cys Ala Asp Ala Ala Lys Met Ala Gly Phe Ala

 -30
 -3
 -1
 +1
 CTC GCC ACC TCT GCG CTC CTC GTC TCC GCG ACC GCG GAG GCG GCG CCC AAG AGG CTG ACC TTC GAC GAG ATC
 Leu Ala Thr Ser Ala Leu Leu Val Ser Gly Ala Thr Ala Glu Gly Ala Pro Lys Arg Leu Thr Phe Asp Glu Ile

 Thylakoid Transfer Domain-----
 TPP
 Mature Protein-----

-10
 CGA AGG AAG ACC TAC ATG GAG GTG AAG GGT ACC GGC ACC GCG GAA
 Gln Ser Lys Thr Tyr Met Glu Val Lys Gly Ser Gly Thr Ala Glu

 KpnI

because the incorporation of ^3H Ala during translation was not efficient enough, or the specific activity of isolated SPP was not great enough.

Section 3:5 Introduction To Pre-sequence Analysis

After the isolation of the 33K cDNA it was becoming apparent that the pre-sequence was responsible for the targeting of polypeptides to the thylakoid lumen. This was based on comparisons with other protein targeting systems, re-targeting studies using the pre-sequence of PC and deletion analysis on the pre-sequence of PC (sections 1:11, 1:8 and 1:6). Shortly after the isolation of the 33K cDNA, H. James isolated the cDNA encoding the 23kDa polypeptide of the OEC (pre-23K). Three approaches were adopted to analyse the pre-sequence of 33K and 23K:-

1. James et al., (1989) had previously shown that SPP and TPP cleaved pre-33K and pre-23K to intermediate and mature forms, respectively. By the use of these peptidases and isolated chloroplast and thylakoid uptake assays it would be possible to show that the sections cleaved by these peptidases contained targeting information.
2. The chemical modification by iodoacetate (IAA) of cysteine residues in the pre-sequences of 33K and 23K (figure 15 and 16) would introduce a large charged group into the pre-sequence that might alter targeting signals. This chemical modification technique is a blunt tool with which to analyse the pre-sequence, but it has had some success in the analysis of the pre-sequences of the stromal precursors of ACP and SSU (Robinson & Ellis, 1984

Figure 16 The DNA sequence and the deduced amino acid sequence of the 23K pre-sequence

The DNA sequence and the deduced amino acid sequence is shown. The TPP and SPP processing sites are indicated as are the deduced envelope and thylakoidal transfer domains (the protein sequence data required for the assignment of the TPP and SPP processing sites was kindly provided by D. Bassham and Dr. P. Elderfield (Warwick)). The numbering refers to the amino acid residues. The cysteine residues are indicated by an asterisk. (DNA sequence provided by H. James (Warwick)).

-70 *
 ATG GCG TCC ACC TCC TGC TTC CTC CAG CAG TCC ACC GCG GCG CTG GCC GCC TCG GCG GCG CTT GCG CCC GCG GTC
 Met Ala Ser Thr Ser Cys Phe Leu His Gln Ser Thr Ala Arg Leu Ala Ala Ser Ala Arg Pro Ala Pro Ala Val
 -----Envelope Transfer Domain-----
 -60
 -50

* -40
 GCG GCG ACC CAG CTT TTC GTC TGC AAG GCG CAG AAG AAT GAC GAG GCT GCA TCT GAC GCT GCC GTC GTC ACC AGC
 Gly Arg Thr Gln Leu Phe Val Cys Lys Ala Gln Lys Asn Asp Glu Ala Ala Ser Asp Ala Ala Val Val Thr Ser

 SPP
 -30

-20
 CCG GCG GCC GCG CTG TCC CTC CTC GCC GGT GCC GCC GCG ATC GCC GTC AAG GTC TCC CCC GCC GCC GCG GCG TAC
 Arg Arg Ala Ala Leu Ser Leu Leu Ala Gly Ala Ala Ile Ala Val Lys Val Ser Pro Ala Ala Ala Tyr
 -----Thylakoid Transfer Domain-----
 -10
 -3
 -1 +1
 TPP

+10
 GGA GAA GCA GCC ACC GTC TTC GCG AAG
 Gly Glu Ala Ala Asn Val Phe Gly Lys
 -----Mature Protein-----

and Musgrove & Robinson, in preparation).

3. The more refined technique of site directed mutagenesis was used to substitute a variety of amino acids at the -1 and -3 residues of the 33K pre-sequence. The -1 and -3 residues were shown by von Heijne *et al.*, (1989) to be small and hydrophobic in all thylakoid lumen targeted polypeptides analysed. It was reasoned that these two residues may play an important role in the TPP cleavage mechanism.

The following sections will consider the use of these three approaches for the analysis of the pre-sequence of the 33K and 23K.

Section 3:6 The Role Of The ETD In Targeting Pre-33K and Pre-23K Across The Chloroplast Envelope

Hageman *et al.*, (1990) have shown by deletion analysis and retargeting studies that the N-terminal section (ETD) of the pre-sequence of PC was required for targeting across the chloroplast envelope, and that the ETD was cleaved by SPP. By virtue of being able to isolate an active stromal extract that could cleave essentially 100% of both pre-33K and pre-23K to their intermediate forms it would be possible to show by a simple processing prior to uptake by chloroplasts that the ETD was required for targeting across the chloroplast envelope.

When either pre-33K or pre-23K translation products were incubated with a stromal extract they were cleaved to their respective intermediate forms (figure 17, lane 1). These intermediates were not taken up by isolated chloroplasts (lane 4) whereas pre-33K and pre-23K translation products were taken up by

Figure 17 The role of the ETD in import into chloroplasts

Panel a

Pre-33K translation mixture (lane T) was incubated with isolated chloroplasts at 25°C for 20mins under illumination, after which the chloroplasts were either washed with 50mM HEPES-KOH pH8.0, 330mM sorbitol (lane 2) or were washed and then treated with 0.2mg/ml of trypsin for 40mins on ice. After incubations these chloroplasts were washed with 50mM HEPES-KOH pH8.0, 330mM sorbitol, 0.2mg/ml of trypsin inhibitor (lane 5). Pre-33K was also treated with a stromal extract for 90mins at 27°C (lane 1) prior to incubation with chloroplasts and either washing (lane 4) or protease treatment (lane 7) as above. Pre-33K was also incubated with intact chloroplasts and a stromal extract and either washed (lane 3) or proteased treated (lane 6) as above. Notation, pre-33K, int-33K and 33K are the precursor, intermediate and mature forms of the 33kDa OEC polypeptide.

Panel b

As panel a except pre-33K was replaced by pre-23K. Notation; pre-23K, int-23K and 23K are the precursor, intermediate and mature forms of the 23kDa OEC polypeptide.



isolated chloroplasts and processed to mature sized products (lane 2), these products were also protease protected within the chloroplast (lane 5). As the chloroplast imports contained stromal extract a control import was also carried out where stroma was added to the import assay (lane 3). In this case pre-33K and pre-23K were both imported into the chloroplast and processed to mature sized products, but to a slightly reduced level. This decrease in uptake level may indicate that the presence of stroma inhibits import to some extent, or that the stromal extract is processing some of the precursor to its intermediate form before it can be imported across the chloroplast envelope. The removal of the ETDs from both pre-33K and pre-23K would appear to prevent targeting across the chloroplast envelope, indicating an absolute requirement in chloroplast import of thylakoid lumenal proteins.

Section 3:7 The Effects Of Iodoacetate On the Import Of Two Thylakoid Lumenal Precursors

Previous work by Musgrove et al., (in preparation) and Robinson & Ellis, (1984) showed that the introduction of a large side chain at or near the SPP cleavage site by carboxymethylation was sufficient to inhibit the processing by SPP of the precursors of two stromal proteins; acyl-carrier protein (ACP) and SSU. Both of these polypeptides contain cysteine residues in their pre-sequences which may be available for carboxymethylation. Analysis of the 33K pre-sequence (section 3:4) revealed two cysteine residues located at positions -37 and -24 (figure 15). A similar sequence analysis of pre-23K revealed two cysteine residues

located at residues -68 and -41 in the pre-sequence (figure 16). The introduction of a large side chain by carboxymethylation into either the pre-sequences of 33K or 23K may disrupt the function of neighbouring targeting signals, such as SPP cleavage, import analysis of these altered precursors would identify any associated import defects. The mechanism of IAA modification of cysteine residues is shown in figure 18.

Many of the models put forward to explain the targeting of precursors to the chloroplast implicate other proteinaceous components such as peptidases, receptors and unfoldases; these other putative components may be sensitive to carboxymethylation. Import analysis could be used to identify these IAA sensitive components required for targeting into the chloroplast. The following section will consider the effect of carboxymethylation on the uptake and intra-organellar targeting of pre-33K and pre-23K.

Section 3:7:a Processing Of Iodoacetate-Modified Pre-23K By A Crude Stromal Fraction And By SPP

James et al., (1989) showed that pre-23K could be processed by a crude stromal extract or partially purified SPP to a protein intermediate in size between the precursor and mature 23K. Figure 19 shows that IAA modification of pre-23K completely blocks cleavage by both SPP and crude stromal extract. The precursor was modified with IAA for 15mins at room temperature prior to processing for 90mins at 27°C with either SPP (lane 3) or a stromal extract (lane 4). The unmodified precursor was converted to an intermediate size when processed for 90mins at 27°C with

Figure 18 The reaction of IAA

Iodoacetic acid (IAA)

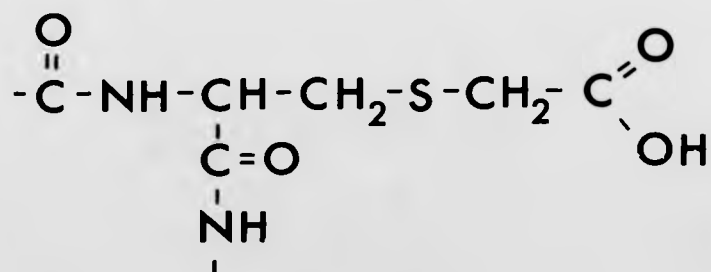
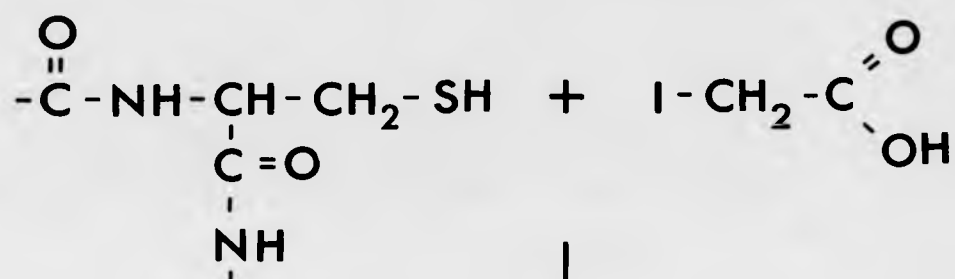
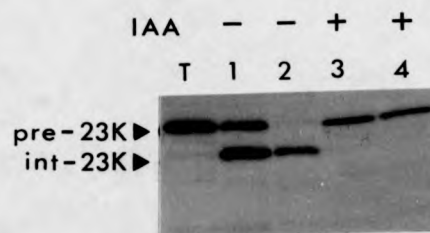


Figure 19 The effect of iodoacetate on processing of pre-23K
by SPP

1 μ l of pre-23K translation mixture (lane T) was incubated with 50mM HEPES pH8.0 at r.t. for 15mins prior to incubation with either partially purified SPP (lane 1) or a crude stromal extract (lane 2) for 90mins at 27°C. Pre-23K was also pre-incubated with 50mM IAA for 15mins at r.t. prior to incubation with SPP (lane 3) or stromal extract (lane 4) for 90mins at 27°C. After incubation the reactions were stopped by the addition of one volume of 2xsample buffer and boiling for 2mins. Samples were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as for figure 17.



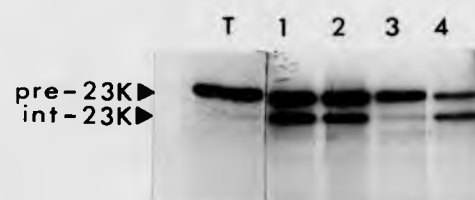
SPP (lane 2) or a stromal extract (lane 3).

In the above experiment IAA was present during the processing incubations and may therefore have carboxymethylated both SPP and pre-23K. To identify the cause of the observed inhibition, partially purified SPP was modified by IAA and then unreacted IAA was removed by dialysis. When this IAA-modified, dialysed, SPP was used to process pre-23K the stromal intermediate was generated (lane 2, figure 20), indicating that the carboxymethylation of SPP does not affect its ability to process the pre-23K. Dialysis of unmodified SPP (lane 4) does not affect its processing activity when compared with unmodified, undialysed SPP (lane 1).

The deduced amino acid sequence of the pre-sequence of pre-23K is shown in figure 16 (sequence data provided by H.James); the cysteine residues are marked (*) as is the SPP cleavage site (based on protein sequence data of the stromal intermediate provided by D. Bassham (Warwick)). The cysteine residue located at the -41 position is only 1 residue away from the SPP cleavage site. This raises the possibility that the introduction of a large, charged side chain by carboxymethylation so close to the SPP cleavage site results in the blockage of the SPP recognition signal and the resultant failure of SPP to process IAA-modified pre-23K, as was also seen for pre-ACP and pre-SSU (Musgrove et al., in prep. and Robinson and Ellis, 1984).

Figure 20 Processing of pre-23K by iodoacetate treated SPP

1 μ l of pre-23K translation mixture (lane T) was incubated with 50mM HEPES pH8.0 at r.t. for 15mins prior to incubation with a partially purified fraction of SPP for 90mins at 27°C (lane 1). Pre-23K was also pre-incubated with 50mM IAA for 15mins at r.t. before incubation with SPP (lane 3). SPP was pre-incubated with 50mM IAA for 15mins at r.t. prior to dialysis at 4°C against 20mM Tris pH8.0; the IAA treated and dialysed SPP was incubated with pre-23K translation mixture for 90mins at 27°C (lane 2). SPP was, also dialysed at 4°C against 20mM Tris pH8.0 prior to incubation with pre-23K translation mixture for 90mins at 27°C (lane 4). After incubations the reactions were stopped by the addition of one volume of 2x sample buffer and boiling for 2mins. Samples were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as for figure 17.



Section 3:7:b Import Of Iodoacetate Modified Pre-23K Into
Isolated Chloroplasts

In the previous section it was shown that the generation of the 23K stromal intermediate could be inhibited by treating the precursor with IAA. This section will consider the fate of IAA-modified pre-23K during import into isolated chloroplasts.

Chloroplasts were isolated and the uptake of IAA-modified and unmodified precursors was carried out, as outlined in section 2:22 and figure 21. Pre-23K was treated at final concentrations of 1mM (lanes 5 and 9), 10mM (lanes 4 and 8), 20mM (lanes 4 and 7) and 50mM (lanes 2 and 6) IAA prior to uptake; the chloroplasts were either washed (lanes 1-5) or were protease treated to remove polypeptides that were not protected by the chloroplast envelope (lanes 6-9). As the concentration of IAA increases, the amount of protein imported into a protease-protected position within the chloroplast decreases (panel a and b). At the highest concentration of IAA used in this experiment (lane 2 and 6), pre-23K was imported into chloroplasts and processed only to a mature sized product. In the previous experiment it was shown that IAA-modification of pre-23K with the same concentration (50mM) of IAA completely blocked cleavage by SPP. These results suggest that the generation of a stromal intermediate is not an absolute requirement for the transport of 23K into the thylakoid lumen as previously suggested by the, "Two step model" (section 1:5). The IAA-modified-23K has been shown to be located in the thylakoid lumen by chloroplast fractionation studies (data not shown).

Figure 21 showed that, as the concentration of IAA increased, there was a decrease in the uptake of pre-23K into

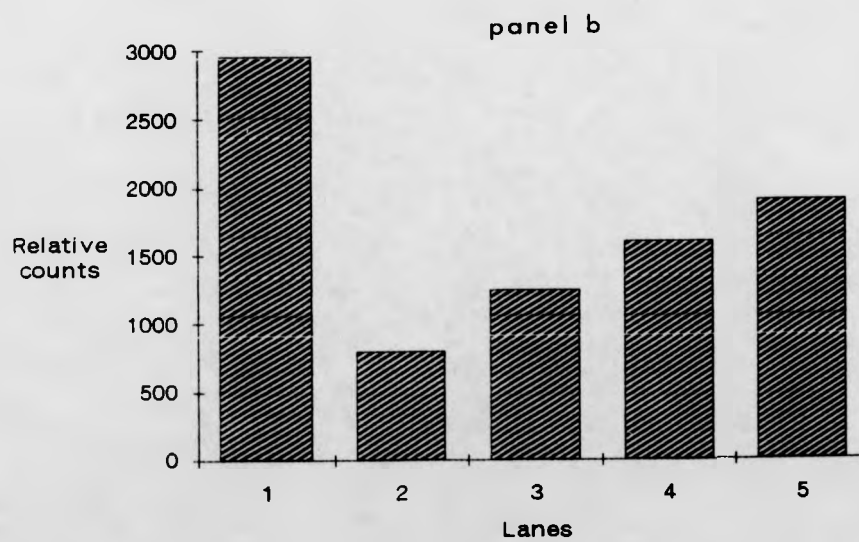
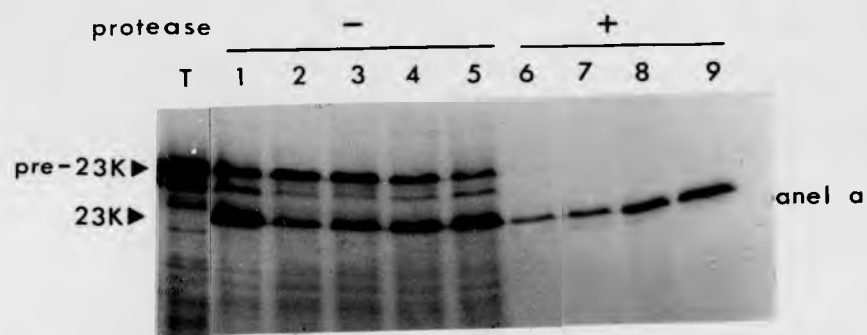
Figure 21 The effect of iodoacetate on the targeting of pre-23K into chloroplasts

Panel a

Pre-23K translation mixture (lane T) was incubated with isolated chloroplasts at 25°C for 20mins under illumination and then washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol and resuspended in 10mM Tris pH8.0 (lane 1). Pre-23K was also pre-incubated with 50(lanes 2 and 6), 20(lanes 3 and 7), 10(lanes 4 and 8) and 1mM(lanes 5 and 9) IAA for 15mins at r.t. before incubation with isolated chloroplasts at 25°C for 20mins under illumination. After incubations the chloroplasts were either washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol and resuspended in 10mM Tris pH8.0 (lanes 2, 3, 4 and 5) or were treated with 0.2mg/ml of trypsin on ice for 40mins after which the chloroplasts were washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol, containing 0.2mg/ml trypsin inhibitor and resuspended in 10mM Tris pH8.0 (lanes 6, 7, 8 and 9). All samples were added to one volume of 2xsample buffer and boiled for 2mins, and were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as in figure 17.

Panel b

The 23K bands present in the non-protease treated lanes (1-5) were quantitated using laser densitometry.



isolated chloroplasts, at 50mM IAA there was a two thirds reduction in the uptake of precursor (panel b. figure 21). This decrease could have been the result of the IAA modification of pre-23K or the unreacted IAA may have affected the uptake apparatus of the isolated chloroplasts. To test this second possibility, intact chloroplasts were isolated and first treated with either 10 or 50mM IAA under uptake conditions before being washed twice to remove any unreacted IAA; these chloroplasts were then used in an uptake experiment with unmodified pre-23K. The result of such an experiment is shown in figure 22, from which it can be seen that import was unaffected by the IAA treatment prior to import (cf. lanes 4 and 6), it may be that low IAA levels stimulate the uptake of pre-23K (lane 5) the reason for this stimulation remains unclear. The result shown in figure 22 when taken together with the observation by Robinson & Ellis, (1986) that IAA can not penetrate across the chloroplast envelope, suggests that there are no IAA sensitive components on the external surface of the chloroplast envelope essential for targeting across the envelope. This reduction in import observed when importing IAA-modified pre-23K into chloroplasts (figure 21) could be due to one of three factors:-

1. An IAA sensitive component present in the wheat germ.
2. An indeterminate effect of IAA on import.
3. A difference in the import of IAA-modified and unmodified pre-23K.

To address these possibilities, IAA-modified pre-23K was dialysed to remove unreacted IAA prior to import into isolated chloroplasts. Figure 23 shows the result of uptake over 2, 5, 10 and 20 minutes of the dialysed, unmodified precursor and

Figure 22 Uptake into iodoacetate treated chloroplasts of pre-23K

Pre-23K translation mixture (lane T) was incubated with isolated chloroplasts at 25°C for 20mins under illumination and was either washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol and resuspended in 10mM Tris pH8.0 (lane 1) or was treated with 0.2mg/ml of trypsin on ice for 40mins after which the chloroplasts were washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol and resuspended in 10mM Tris pH8.0 containing 0.2mg/ml trypsin inhibitor (lane 4). Isolated chloroplasts were also pre-incubated with either 10mM (lane 2 and 5) or 50mM IAA (lane 3 and 6) and then washed twice in 50mM HEPES-KOH pH8 , 330mM sorbitol prior to incubation with pre-23K at 25°C for 20mins under illumination. After incubation in the light these chloroplasts were either washed (lanes 2 and 3) or protease treated (lanes 5 and 6). All samples were added to one volume of sample buffer and boiled for 2mins after being resuspended in 10mM Tris pH8.0. Samples were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as in figure 17 and - untreated chloroplasts; + 10mM IAA treated chloroplasts; ++ 50mM IAA treated chloroplasts.

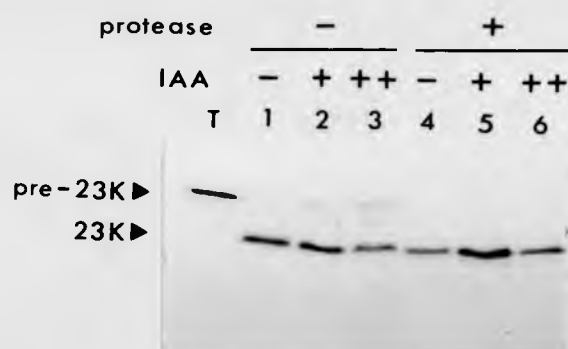


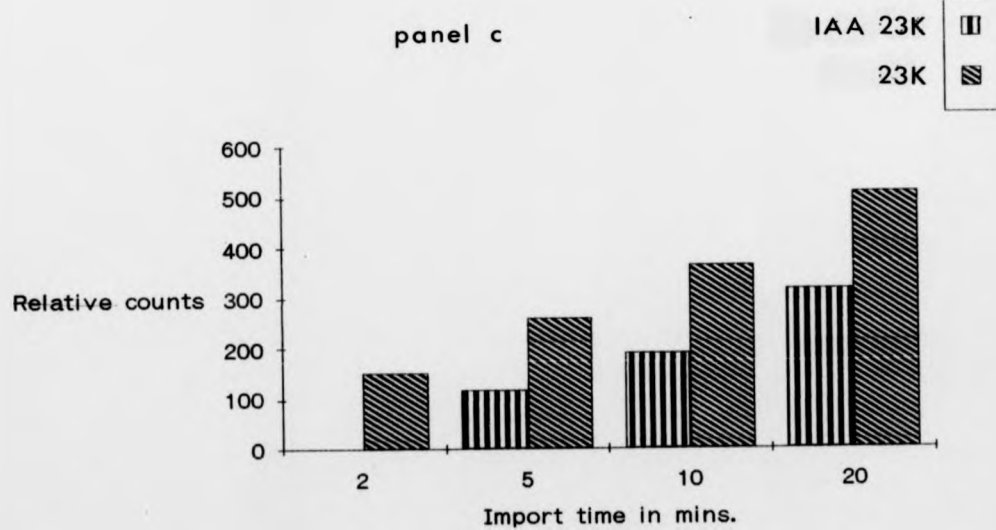
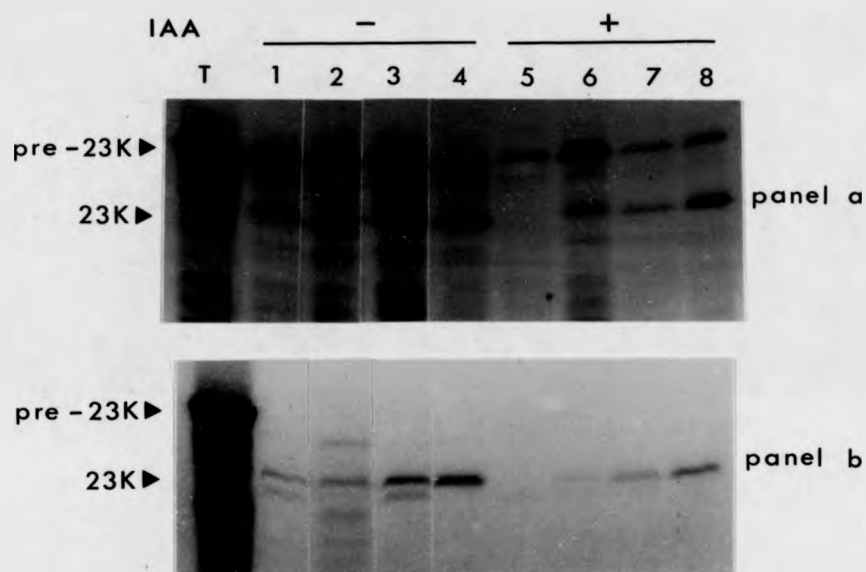
Figure 23 Time course of import of iodoacetate modified pre-23K into chloroplasts

Panel a and b

Pre-23K translation mixture (lane T) was either pre-incubated with 50mM IAA pH8.0 (lanes 5-8) or was pre-incubated with 50mM HEPES-KOH pH8.0 (1-4), at r.t. for 15 mins. prior to dialysis against 4dm⁻³ of 10mM HEPES pH8.0 at 4°C for 4hrs. The above precursors were incubated with intact chloroplasts at 25°C under illumination for 2 (lanes 1 and 5), 5 (lanes 2 and 6), 10 (lanes 3 and 7) and 20 mins (lanes 4 and 8). After incubations the chloroplasts were either washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol and resuspended in 10mM Tris pH8.0 (panel a) or were treated with 0.2mg/ml of trypsin on ice for 40mins after which the chloroplasts were washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol, containing 0.2mg/ml trypsin inhibitor and resuspended in 10mM Tris pH8.0 (panel b). All samples were added to one volume of sample buffer and boiled for 2mins., and were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as in figure 17.

Panel c

The intensity of the mature 23K bands present in panel a were quantitated by laser densitometry.



dialysed, IAA-modified precursor. The rate of uptake of the IAA-modified and dialysed precursor was delayed when compared with that of unmodified, dialysed precursor (cf. lane 1-4 and 5-8 and panel c). This delay in the rate of uptake may suggest a difference in the kinetics of targeting of IAA-modified and unmodified precursor, but may also suggest an IAA sensitive component is present in the wheat germ used to generate the pre-23K. In an attempt to identify the putative enhancing factor present in the wheat germ, the wheat germ was treated with IAA prior to translation. This IAA-modified wheat germ was, however, inactive when used in the in vitro translation system, (data not shown).

Section 3:7:c Uptake Of Iodoacetate Modified Pre-23K Into Isolated Thylakoids

In vitro chloroplast import and processing studies on IAA-modified pre-23K suggest that the full precursor can be targeted across the thylakoid membrane, and not just the intermediate as postulated by the two step model. The use of an isolated chloroplast uptake assay, however, can not exclude the possibility that an intermediate is generated in the stromal phase, although this would be unlikely as a stromal fraction is unable to process IAA modified pre-23K to its intermediate form. Mould et al., (1991) have developed an isolated thylakoid vesicle uptake system which would allow the targeting of IAA-modified pre-23K across the thylakoid membrane to be addressed directly without the complication of targeting across the chloroplast envelope and intra-organelar routing through the stromal phase.

The following section will consider the targeting of IAA-modified pre-23K across isolated thylakoid vesicles.

Figure 24 shows that when pre-23K was modified by IAA prior to import into isolated thylakoid vesicles, the IAA-modified precursor was targeted into a protease protected position within the thylakoid lumen (lane 6). A crude stromal extract could not process pre-23K modified with IAA (lane 2) but could process unmodified precursor to a stromal intermediate (lane 1), when taken with the result shown in lane 6 this suggests that the complete precursor can target successfully across the thylakoid membrane. The efficiency of targeting IAA-modified pre-23K (lanes 4 and 6) when compared with the targeting of unmodified precursor (lanes 3 and 5) was reduced (also see figure 26). The efficiency of targeting was judged at a single time point and as such does not allow conclusions about the rates of import to be drawn. The reduction in efficiency seen in figure 24 may result from:-

1. A difference in the efficiency of import of pre-23K and int-23K.
2. An IAA sensitive import factor present in the stroma added to the import or the wheat germ used to generate the pre-23K polypeptide.
3. Some indirect effect of IAA on the thylakoid import mechanism.

In an attempt to resolve the above possibilities, the IAA-modified and unmodified pre-23K were dialysed to remove unreacted IAA, prior to uptake into isolated thylakoid vesicles. Figure 25 shows the result of such an experiment, the IAA-modified (lanes 1 and 2) and unmodified (lanes 3 and 4) pre-23K were imported into

Figure 24 Effect of iodoacetate on the import of pre-23K into thylakoids

Pre-23K translation mixture (lane T) was incubated with a stromal extract for 90mins at 27°C (lane 1). The translation mixture was also pre-incubated with IAA at r.t. for 15mins before incubation with a stromal extract at 27°C for 90mins (lane 2). Isolated thylakoids were incubated at 25°C under illumination with pre-23K that had either been pre-incubated with 50mM HEPES pH8.0 at r.t. for 15mins (lanes 3 and 5) or pre-23K that had been pre-incubated with 50mM IAA (in 50mM HEPES pH8.0) at r.t. for 15mins (lanes 4 and 6). After incubation in the light the thylakoids were either washed in 10mM HEPES pH8.0 (lanes 3 and 4) or were treated with 0.05mg/ml proteinase K for 30mins on ice followed by the addition of 50µl of 7mg/ml of PMSF followed by washing in 10mM Tris-Cl pH7.6 (lanes 5 and 6). All samples were incubated with 1 volume of 2xsample buffer and boiled for 2mins., and were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as in figure 17.

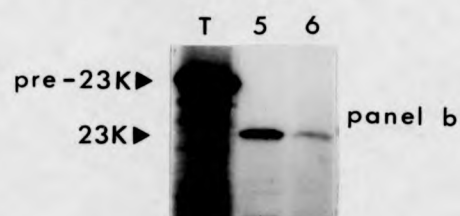
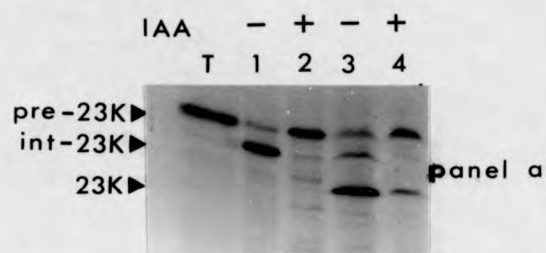
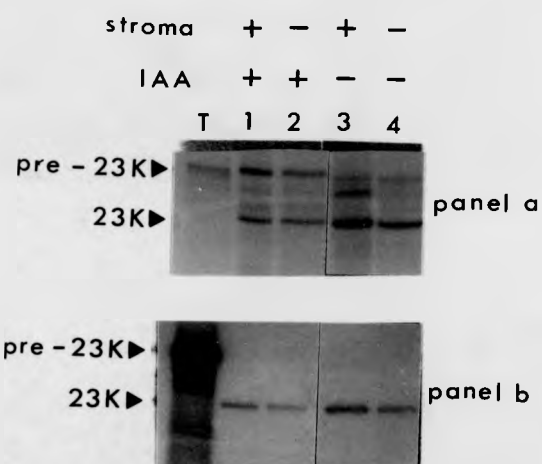


Figure 25 The uptake into thylakoids of iodoacetate modified pre-23K

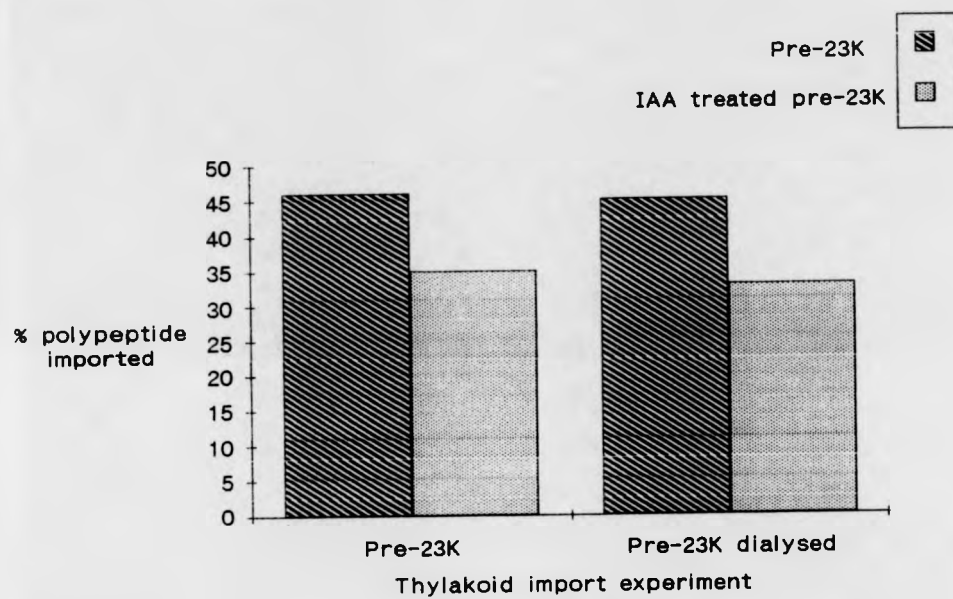
Pre-23K translation mixture (lane T) was dialysed against 4dm^{-3} of 50mM HEPES-KOH pH8.0 at 4°C prior to being incubated at 25°C for 20mins under illumination with thylakoids in the presence (lane 3) and absence (lane 4) of additional stromal extract. Pre-23K translation mixture was also pre-incubated with 50mM IAA pH8.0 prior to being dialysed against 4dm^{-3} of 50mM HEPES-KOH pH8.0 at 4°C . The IAA treated and dialysed translation mixture was then incubated at 25°C for 20mins under illumination with thylakoids in the presence (lane 1) and absence (lane 2) of additional stromal extract. The thylakoids after illumination were either added directly to 1 volume of 2x sample buffer (panel a) or were treated with 0.05mg/ml of proteinase K for 30mins on ice. The proteinase was inactivated by the addition of 50ul of 7mg/ml of PMSF followed by washing in 10mM Tris-Cl pH7.6 before the addition of 1 volume of 2x sample buffer (panel b). Notation as for figure 17.



a protease protected position within the thylakoids, IAA-modified and unmodified pre-23K were not imported with the same efficiencies (see figure 26). The difference in the uptake efficiencies of IAA-modified and unmodified pre-23K which had been dialysed suggest that there is a difference in the uptake of the complete precursor and the stromal intermediate; as dialysis of the IAA-modified precursor excludes the possibility that this difference in import efficiency was an indirect effect of IAA on import of IAA inactivation of a stromal component. This difference in import efficiency may simply reflect an alteration in precursor conformation resulting in altered import characteristics or the presence of the ETD may impair the efficiency of import across the thylakoid membrane. A comparison of the % polypeptide imported in the experiments under taken using dialysed or non-dialysed precursor (figures 24 and 25) reveals that dialysis of pre-23K or IAA-modified pre-23K had no effect on its import efficiency (figure 26); this allows the exclusions of an IAA sensitive component present in the stroma or exposed on the thylakoid membrane surface. It is, however, interesting to noted that there is a 10% reduction in the % polypeptide imported between pre-23K and IAA-modified pre-23K in both undialysed and dialysed precursor, suggesting that IAA does not have an indirect effect on import into thylakoids, and the 10% reduction seen is most probably the result of differences in the import of pre-23K and IAA-modified pre-23K. It was not possible to discount the role of an IAA sensitive component present in the wheat germ.

Figure 26 The efficiency of import into isolated thylakoids:
the effect of dialysis and iodoacetate treatments

The intensity of the bands present in the thylakoid import experiments represented in figures 24 and 25 were quantitated by laser densitometry. The effect of IAA treatment of pre-23K are shown, as are the effects of the removal of free IAA by dialysis on import into thylakoids. The efficiency of import is represented by the mature polypeptide present in the non-protease treated fraction compared to the total polypeptide (precursor, intermediate and mature forms) present in the fraction.



Section 3:7:d The Effects Of Iodoacetate Modification Of Pre-33K: Introduction

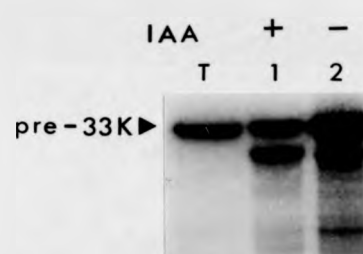
A similar approach to the analysis of the 23K pre-sequence was adopted for the analysis of the 33K pre-sequence. The determination of the 33K pre-sequence identified two cysteine residues (section 3:4), and tests were carried out to determine whether the carboxymethylation of these residues affects import or processing of this precursor.

Section 3:7:e Processing Of Iodoacetate Modified Pre-33K By Partially Purified SPP

Previous work by James et al., (1989) demonstrated that pre-33K is processed to a stromal intermediate by SPP (section 3:3 and figure 27, lane 2). When pre-33K was modified by IAA prior to processing by SPP an intermediate was also generated (figure 27, lane 1); this intermediate, however, had a reduced mobility when compared to the stromal intermediate (lane 2). This reduction in mobility was either the result of the addition of large side chains following carboxymethylation or aberrant cleavage by SPP. In an attempt to resolve this point the intermediates resulting from cleavage with SPP of pre-33K and IAA modified pre-33K (labelled with ^3H Ala, instead of ^{35}S Met) were sent to Dr. B. Dunbar (Aberdeen) for protein sequencing. This attempt was, however, unsuccessful. Some recent work by D. Bassham (Warwick) using freshly prepared SPP and ^3H Leu or ^3H Phe labelled pre-33K and IAA-modified pre-33K has shown that SPP cleaves at the same

Figure 27 Processing of pre-33K by SPP: the effect of iodoacetate

Pre-33K translation mixture (lane T) was pre-incubated with 50mM iodoacetate at r.t. for 15mins before being incubated with SPP at 27°C for 90mins (lane 1). Pre-23K translation mixture was also pre-incubated with 50mM HEPES-KOH pH8.0 at r.t for 15mins prior to incubation with SPP for 90mins at 27°C (lane 3). After incubations the reactions were stopped by the addition of one volume of 2x sample buffer and boiling for 2mins. Samples were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as in figure 17.



position. Thus the alteration in mobility seen in figure 27 is the result of the addition of large side chains resulting from the carboxymethylation of the precursor. Thus IAA-modification of pre-33K does not affect the site at which SPP cleaves. It is, however, notable that the carboxymethylation of pre-33K leads to an increased rate of processing by SPP, the reason for this increased rate of cleavage remains unclear. Carboxymethylation of pre-33K does not alter the site of SPP cleavage unlike the carboxymethylation of pre-23K where the SPP cleavage site was blocked.

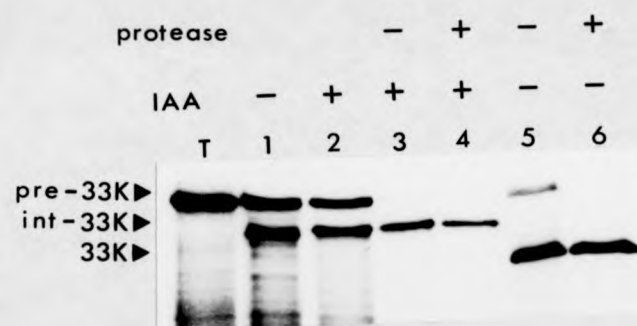
Section 3:7:f Import Of Iodoacetate Modified Pre-33K Into
Isolated Chloroplasts

The IAA-modified pre-33K has been analysed with respect to processing by SPP (above); in this section it will be analysed with respect to its uptake by isolated intact chloroplasts.

In section 3:3 it was shown that pre-33K was imported into chloroplasts and routed to the thylakoid lumen. Figure 28 shows the uptake by chloroplasts of IAA-modified pre-33K; the modified precursor was successfully targeted into the chloroplasts (lanes 3 and 4) but was not processed to the mature sized polypeptide observed when unmodified polypeptide was targeted into chloroplasts (lanes 5 and 6). Instead the polypeptide was of the same mobility as that produced by the incubation of IAA-modified pre-33K with SPP (lane 2). The import study shown in figure 28 indicates that IAA-modified pre-33K was targeted into the chloroplast where it was processed to the stromal intermediate;

Figure 28 Uptake by chloroplasts of pre-33K: the effect of iodoacetate

Pre-33K translation mixture (lane T) was incubated with SPP for 90 mins at 27°C (lane 1), the translation mixture was also pre-incubated with 50mM IAA pH8.0 before being incubated with SPP for 90mins at 27°C (lane 2). IAA pre-incubated pre-23K was also incubated with intact chloroplasts at 25°C for 20mins under illumination, after which the chloroplasts were either washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol and resuspended in 10mM Tris pH8.0 (lane 3) or were washed and then treated with 0.2mg/ml of trypsin on ice for 40mins and then were washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol, containing 0.2mg/ml trypsin inhibitor and resuspended in 10mM Tris pH8.0 (lane 5). Pre-23K which had been pre-incubated with 50mM HEPES pH8.0 for 15mins at r.t. was also incubated with intact chloroplasts at 25°C for 20mins under illumination, after which the chloroplasts were either washed (lane 5) or protease treated (lane 6) as above. All samples were added to one volume of sample buffer and boiled for 2mins., and were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as in figure 17.



it does not indicate whether this polypeptide was also routed to the thylakoid lumen or remains in the stromal phase. The chloroplast fractionation study shown in figure 29 shows that IAA-modified stromal intermediate was in fact targeted only as far as the stromal compartment (lane 4), and not into the thylakoid lumen (lane 5). The inability of IAA-modified stromal intermediate to target to the thylakoid lumen probably reflects a failure of the modified polypeptide to recognise the putative receptor located in the thylakoid membrane as there was little binding of the imported polypeptide to the thylakoid membrane (lane 5, Figure 29). A failure in import at a later stage of translocation (translocator or thylakoid peptidase) would either result in a thylakoid membrane-bound or thylakoid lumen-located polypeptide, neither of which were observed. It is conceivable that the import blockage could occur before binding to the thylakoid receptor; this would presumably involve a chaperone-like function.

In both of the import experiments described above IAA was present at 5mM during the incubations, raising the possibility that IAA was affecting the chloroplast import apparatus resulting in the default targeting of pre-33K across the chloroplast envelope. Such default import mechanisms have been invoked for the mistargeting of rubisco SSU across the mitochondrial envelope. To test this possibility, chloroplasts were treated with 10mM and 50mM IAA, and then washed twice before being used to import pre-33K. As seen in Figure 30, these IAA treated chloroplasts import pre-33K to a protease protected mature size protein indicating that the import apparatus was not affected by IAA being present during import. It would appear that the failure

Figure 29 Localization of imported 33K: the effect of iodoacetate

Pre-33K translation mixture (lane T) was incubated with isolated chloroplasts at 25°C for 20mins under illumination after which the chloroplasts were washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol and lysed in 10mM Tris pH8.0 (lane 1). Pre-33K translation mixture was also pre-incubated with 50mM IAA pH8.0 at r.t. for 15mins before incubation with intact chloroplasts for 20mins at 25°C under illumination; after incubation the chloroplasts were either washed with 50mM HEPES-KOH pH8.0, 330mM sorbitol (lane 2) or were washed and then treated with 0.2mg/ml of trypsin for 40mins on ice. After incubations, these chloroplasts were washed with 50mM HEPES-KOH pH8.0, 330mM Sorbitol, containing 0.2mg/ml of trypsin inhibitor, and either analysed directly (lane 3) or after lysis with 10mM Tris pH7.6 and fractionation into stromal (lane 4) and thylakoidal (lane 5) fractions. All samples were added to one volume of 2x sample buffer and boiled for 2mins., and were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as in figure 17.

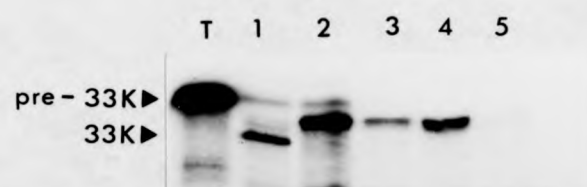


Figure 30 Uptake into iodoacetate treated chloroplasts of pre-33K

Pre-33K translation mixture (lane 9) was incubated with isolated chloroplasts at 25°C for 20mins under illumination, after which the chloroplasts were either washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol and resuspended in 10mM Tris pH8.0 (lane 8) or were treated with 0.2mg/ml of trypsin on ice for 40mins after which the chloroplasts were washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol, containing 0.2mg/ml of trypsin inhibitor and resuspended in 10mM Tris pH8.0 (lane 4). IAA-modified pre33K was also incubated with isolated chloroplasts at 25°C for 20mins under illumination after which the organelles were either washed (lane 7) or protease treated (lane 3) as above. Isolated chloroplasts were also pre-incubated with either 10mM (lanes 6 and 2) or 50mM IAA (lanes 5 and 1) and then washed twice in 50mM HEPES-KOH pH8.0, 330mM sorbitol prior to incubation with pre-23K at 25°C for 20mins under illumination. After incubation in the light these chloroplasts were either washed (lanes 6 and 5) or protease treated (lanes 2 and 1) as above. All samples were added to one volume of sample buffer and boiled for 2mins., and were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as in figure 17 and - untreated chloroplasts; + 10mM IAA treated chloroplasts; ++ 50mM IAA treated chloroplasts.

+	+	+	+	-	-	-	-	
++	+	•	-	++	+	•	-	
1	2	3	4	5	6	7	8	9

Protease
IAA

◀ pre-33 kDa
◀ int-33 kDa
◀ 33 kDa

• IAA modified precursor

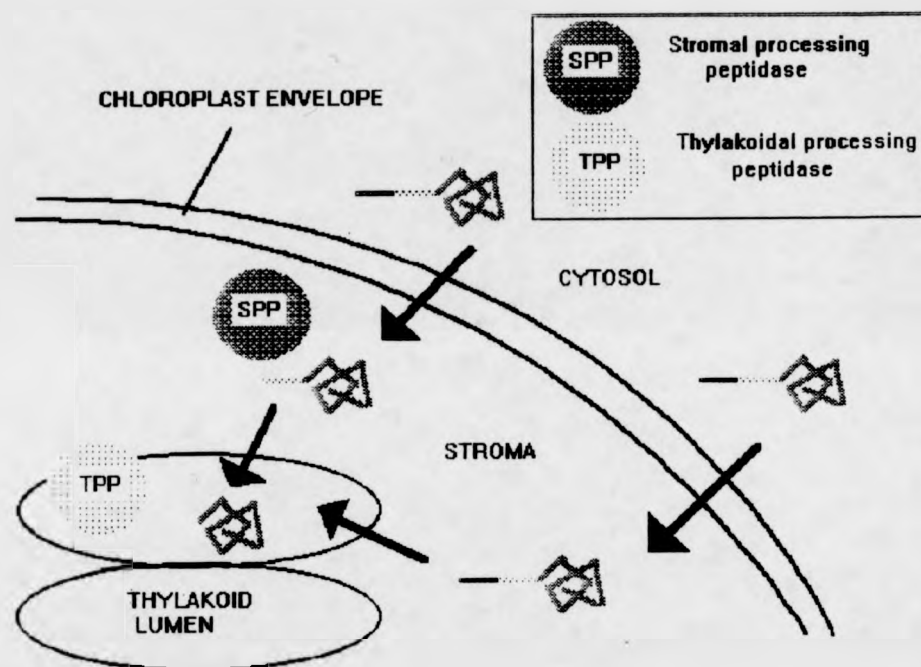
of IAA modified pre-33K to target to the thylakoid lumen results from the modification of the precursor's cysteine residues. The possibility also exists that IAA may be affecting translocation factors in the wheat germ used in translations, but this cannot be tested since IAA modification of wheat germ inactivates its capacity for translocation (section 3:7:b). The failure to target to the thylakoid lumen most probably reflects the inability of stromal intermediate to recognise the thylakoidal import apparatus.

Section 3:7:g Summary Of Iodoacetate Modification Of Pre-23K
And Pre-33K

When pre-23K was modified with IAA the SPP cleavage site was blocked. This modified precursor, however, was successfully transported to the the thylakoid lumen where the complete pre-sequence was cleaved by TPP, this is first demonstration of targeting of the complete precursor across the thylakoid membrane of a nuclear encoded thylakoid lumen targeted polypeptide. The targeting of the full 23K precursor form across the thylakoid membrane also provides the first demonstration of an additional route to that outlined in the two step model for the targeting of nuclear encoded proteins to the thylakoid lumen (figure 31). The ability of the pre-23K to cross the thylakoid membrane both in the chloroplast and in the thylakoid uptake experiments indicates that the thylakoid transfer domain can act as an internal targeting signal. A non-cleavable, internal targeting signal has previously been identified for the integration of LHCP into the

Figure 31 A schematic representation of protein transport into chloroplasts and intra-organellar routing to the thylakoid lumen

The pre-23K protein is synthesised in the cytoplasm as a precursor with an N-terminal targeting signal. The precursor is then imported across the chloroplast envelope into the stroma where in the "two step model" the N-terminal section of the targeting signal (the envelope transfer domain) is removed by the stromal processing peptidase (SPP), and the stromal intermediate is targeted across the thylakoid membrane where the remaining section of the targeting signal (the thylakoid transfer domain) is removed by the thylakoidal processing peptidase (TPP). In the "one step model" the precursor is targeted across the chloroplast envelope and then across the thylakoid membrane where the targeting signal is removed by the TPP in a single step.



thylakoid membrane (Lamppa, 1988 and Viitanen et al., 1988), but the above data represent the first identification of an internal targeting signal directing transport into the thylakoid lumen.

The targeting efficiency across the thylakoid membrane was reduced by 10% (figure 26). This reduction in efficiency may reflect a decrease in chaperonin, receptor, translocase or peptidase function as a result of the modification of cysteine residues in pre-23K or results from the presence of the ETD. The ETD is required for targeting across the chloroplast envelope, but its presence during import across the thylakoid membrane may result in a decrease in efficiency of targeting. The ETD may interfere with the interaction of the TTD with possible chaperonines, receptors, translocation machinery or thylakoid peptidase function. The decrease in efficiency of targeting seen may also result from changes caused in protein structure as a result of the IAA modification of cysteine residues in pre-23K. The inability of pre-23K to target across the thylakoid membrane as efficiently as the stromal intermediate may indicate why the stromal cleavage event has been retained.

Some of the decrease in targeting efficiency across the chloroplast envelope may be due to the inactivation of an IAA sensitive component present in the wheat germ of the translation. This component may have a function in maintaining the import competence of the precursor, but this has not been shown to be the case yet. The reduction in import efficiency across the thylakoid membrane may also be due to an IAA sensitive component present in the wheat germ of the translation.

Analysis of the complete pre-23K sequence (H.James, personal communication (Warwick)) reveals that there are 3 cysteine

residues in the precursor, two of which are located in the pre-sequence. The cysteine residue at position -41 is located 1 residue from the SPP cleavage site (figure 16). It would be tempting to speculate that the introduction of a large side chain (by carboxymethylation) at this cysteine residue results in the alteration of the SPP cleavage signal so that the SPP can no longer recognise the cleavage site. IAA-modification of pre-23K blocks cleavage to the stromal intermediate but does not prevent targeting to the thylakoid lumen. This indicates that there is not a prerequisite for a stromal intermediate in targeting to the thylakoid lumen.

The results of IAA modification of pre-33K were completely different to those of the IAA modification of pre-23K. The IAA modification of pre-33K does not inhibit pre-33K cleavage, and in fact enhances cleavage for reasons that remain unclear. However, this modification of pre-33K does block targeting across the thylakoid membrane. It is assumed that modification of the targeting signal inactivates some part of the transport system across the thylakoid membrane. The only cysteine residue in the TTD is located at position -24, this region is required in targeting across the thylakoid membrane. It should be noted that there are other cysteine residues located in the mature 33K polypeptide and carboxymethylation of these could result in the import block seen above, although this is less likely.

Section 3:8 Analysis Of The TPP Cleavage Site Using
Mutagenesis

At the primary sequence level one of the two conserved features of cytoplasmically synthesised precursors targeted to the thylakoid lumen, is the presence of small hydrophobic residues at the -3 and -1 positions (section 1:2:c). The proximity of this conserved motif to the TPP cleavage site raises the possibility that it forms part of the recognition signal for TPP cleavage, although there is no biochemical data to support this assumption. One of the more interesting aspects of protein targeting to emerge recently has been the similarity in reaction specificity of TPP and prokaryotic signal peptidase I (Leader peptidase (LEP)). This similarity was clearly demonstrated by Halpin et al., (1989) who showed that LEP (E.coli signal peptidase I) would cleave thylakoidal pre-sequences and that TPP would cleave bacterial export signals and probably eukaryotic E.R. targeting signals. The targeting signals of proteins exported from E.coli (which are cleaved by LEP) show similarity to TTDs in containing small hydrophobic residues at their -1 and -3 positions. Fikes et al., (1990) has clearly demonstrated the importance of the -1 and -3 position in the cleavage of the E.coli precursor of maltose binding protein (pre-MBP) by LEP. When the above are considered together it would seem that the -1 and -3 residues of TTDs form part of the TPP cleavage site.

The aims of this part of my work were two fold:

1. To investigate the reaction mechanism of TPP, by substituting a variety of amino acids residues at the -1 and -3 positions of pre-33K.

2. To carry out a detailed comparison of the substrate requirements of pre-33K at the -3 and -1 positions for TPP and LEP cleavage.

The approach taken was to substitute a number of residues at the -3 and -1 positions (by site directed mutagenesis) and to assess the maturation of these mutant precursors on their import into chloroplasts. In addition a direct comparison of the substrate requirements of LEP and TPP were made by the expression of the mutants in E.coli, since it was known that the pre-33K is exported into the periplasmic space (Meadows et al., in press; & Seidler and Michel, 1990). An indirect comparison of the substrate requirements of TPP and LEP can be made by comparing the substrate requirement of pre-33K for TPP (above) and that of pre-MBP for LEP (Fikes et al., 1990).

Section 3:8:a Generation Of Oligonucleotide-Directed Mutations

The oligonucleotides required for the mutagenesis reaction were synthesised as indicated in section 2:20:a and their sequences are shown below:

GGG CGC CCC CTC CGC CGT CGC GCC GGA	Original
--- --- --- --- CAG --- --- --- ---	-1Leu
--- --- --- --- CTT --- --- --- ---	-1Lys
--- --- --- --- CTC --- --- --- ---	-1Glu
--- --- --- --- GGT --- --- --- ---	-1Thr
--- --- --- --- CGA --- --- --- ---	-1Ser
--- --- --- --- CCC --- --- --- ---	-1Gly
CCC CTC CGC CGT CGC GCC GGA GAC GAG	Original
--- --- --- --- CAG --- --- --- ---	-3Leu[1]
CGC --- --- --- --- CAG --- --- --- --- GAG	-3Leu[2]
--- --- --- --- CAA --- --- --- ---	-3Leu[3]
--- --- --- --- CTT --- --- --- ---	-3Lys
--- --- --- --- CTC --- --- --- ---	-3Glu
--- --- --- --- CAC --- --- --- ---	-3Val

All oligonucleotides were phenol / chloroform extracted before being resuspended in water to a final concentration of 20nmol/ml. These oligonucleotides were synthesised lacking their 5' phosphates which are required for the completion of synthesis of the mutant strand in the mutagenesis reaction. The oligonucleotides were phosphorylated as in section 2:20:b. The phosphorylated oligonucleotides were then used directly in the mutagenesis reaction as described in section 2:20:d to generate mutations. The oligonucleotides were annealed to ssDNA of the vector M13mp18-33-150. This vector contains the 150bp Kpn1 / Pst1 insert from p33K-2 which includes the -3Ala X -1Ala site; it was constructed as outlined in section 2.20.c.

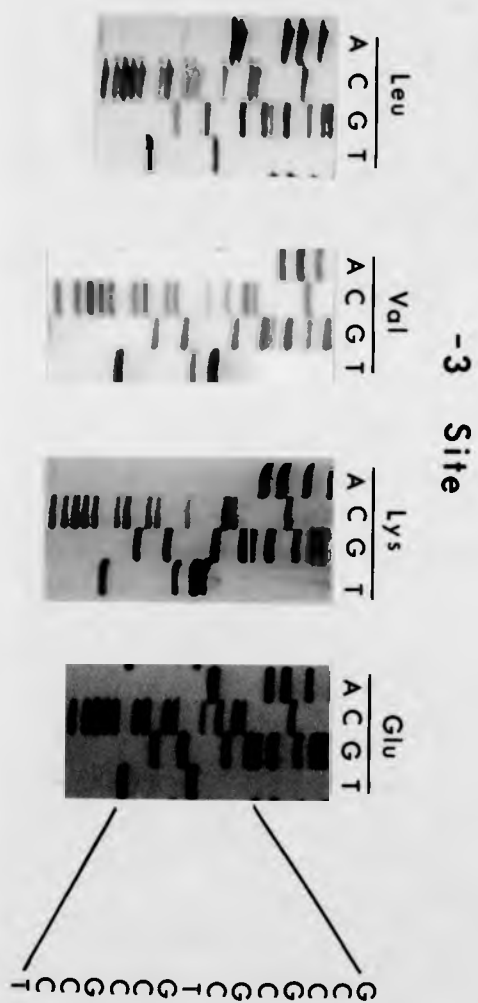
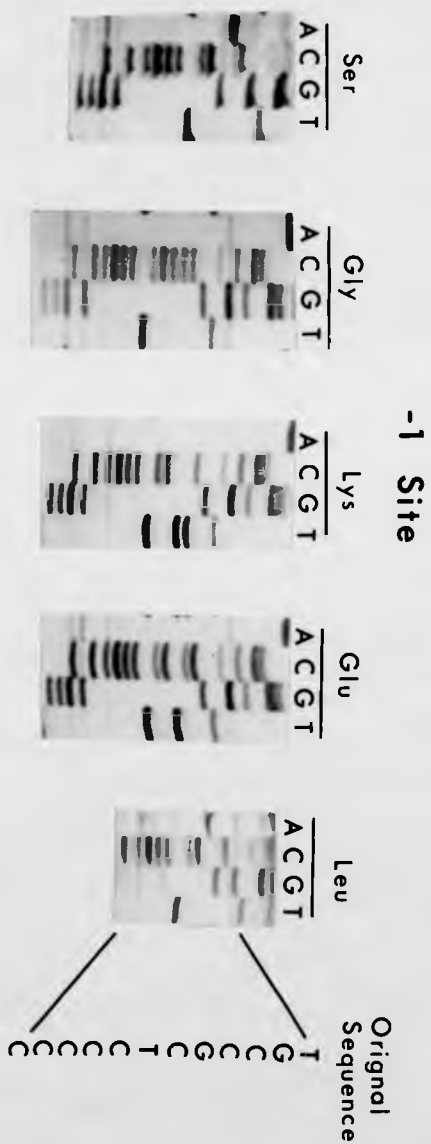
Confirmation that the desired mutation had been created was carried out by the sequencing of the DNA extracted from the

plaques produced at the end of the mutagenesis reaction. Figure 32 shows the sequence data generated for the successful mutagenesis reactions undertaken. As can be seen from figure 32 most of the mutants gave the expected sequence at either the -1 or -3 positions. The -3Leu[1] mutation, however did not give the expected sequence. This may have resulted from the high GC to AT ratio of the oligonucleotide used; in an attempt to reduce this ratio the oligonucleotide was extended by 6bp (-3Leu[2]), this oligonucleotide did not give the expected sequence. An attempt was also made to reduce this ratio in the oligonucleotide by changing the codon used to code for leucine from GAC to AAC. When this oligonucleotide (-3Leu[3]) was used in the mutagenesis reaction the -3Ala was successfully changed to a -3leu. The complete Kpn1 / Pst1 insert of M13mp18-33-150 was sequenced for each mutation to check that the oligonucleotide had not annealed elsewhere and caused additional mutations. In all cases except -Leu[1] and -3Leu[2] only the expected mutation was seen.

Once the mutations had been generated in the 150bp Kpn1 / Pst1 insert they were inserted into the transcription vector p33K-2* which had been linearised by digestion with Kpn1 and Pst1 so releasing the non-mutated 150bp Kpn1 / Pst1 insert as outlined in section 2:20:e. The vector p33K-2* is derived from the transcription / translation vector p33K-2 (section 2:20:e); it lacks the Sac1 - Sph1 region of the polylinker of p33K-2. The above cloning procedure results in the replacement of a mutated Kpn1 / Pst1 insert for a non-mutated Kpn1 / Pst1 insert. In order to confirm that the above subcloning was successful a single colony was picked and DNA isolated from it, the Kpn1 / Pst1 insert was released and subcloned into M13mp18 which had been

Figure 32 Sequence confirmation of the mutant pre-33Ks

The -1 and -3 mutants were created by site directed oligonucleotide mutagenesis as described under "Results and Discussion" (section 3:5:a). Confirmation that the desired mutation had been created was by the sequencing as described under "Results and Discussion" (section 3:5:a). The non-mutated 5'-3' DNA sequence of the cDNA encoding pre-33K for the -1 and -3 regions is shown.



linearised with Kpn1 and Pst1 as in section 2:20:f. When these Kpn1 / Pst1 inserts were sequenced all contained the expected mutations.

Section 3:8:b Processing By SPP and TPP Of The -3 and -1
Mutants

The transcription vectors p33K-2*(-1Leu), p33K-2*(-31eu), etc. when transcribed and translated as in sections 2:23 and 2:24 generate 42kDa precursor proteins when analysed by SDS-PAGE and fluorography. These precursors were of the same size as that generated from non-mutated p33K-2. The mutant precursors were each incubated with SPP or TPP.

All of the precursors generated above were processed by SPP to their stromal intermediate forms as shown in figure 33. This indicates that none of the amino acid substitutions created result in gross structural alterations in the precursor that would be likely to affect SPP cleavage. Figure 34 shows the processing by TPP of the pre-33Ks containing the ten mutations generated above as compared to processing by TPP of the non-mutated pre-33K. Wild type pre-33K was processed by TPP to a mature size as shown previously by Kirwin *et al.*, (1989)(lane 2); of the ten mutations created at the TPP cleavage site only the -3Val was processed (lane 9). The TPP processed -3Val to a mature sized protein with similar efficiency as that of non-mutant pre-33K, as judged by analysis by SDS-PAGE followed by fluorography and densitometric determination of mature polypeptide (figure 34, panels a and b). Clearly, recognition by TPP of pre-33K is very sensitive to changes at either the -1 or -3 positions and will

Figure 33 Cleavage of the pre-33K mutants by stromal processing
peptidase

Non-mutated and mutated pre-33Ks were synthesised by in vitro transcription-translation as described under "Materials and Methods" (sections 2:23 and 2:24). 1 μ l aliquots of translation mixture were incubated with 20 μ l of partially purified SPP activity for 90mins at 27°C. Lanes T and 1: non-mutated pre-33K before and after incubation with SPP. Lanes 2-11, incubation of SPP with -3Val, Glu, Lys, Leu and -1Gly, Ser, Glu, Lys, Leu and Thr, respectively. Pre-33K and int-33K, precursor and intermediate forms of 33K, respectively.

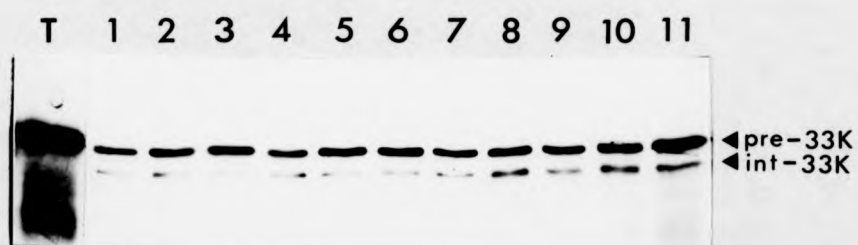


Figure 34 Cleavage of the pre-33K mutant proteins by thylakoidal processing peptidase

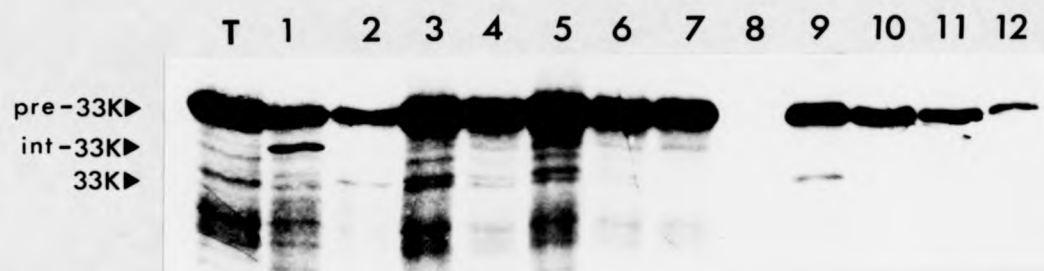
Panel a

Non-mutated and mutated pre-33Ks were synthesised by in vitro transcription-translation as described under "Materials and Methods" (sections 2:23 and 2:24). 1 μ l aliquots of translation mixture were incubated with 20 μ l of partially purified TPP activity for 90mins at 27°C. Lanes T and 2: non-mutated pre-33K before and after incubation with TPP. Lane 1, 1 μ l of non-mutated pre-33K incubated with 20 μ l of SPP activity for 90mins at 27°C. Lanes 3-12, incubation of TPP with -1Leu, Lys, Glu, Thr, -3Glu, Lys, Val, Leu, -1Ser and Thr respectively. Pre-33K, int-33K and 33K, precursor, intermediate and mature forms of 33K, respectively. The -3Lys did not translate efficiently in this experiment but was not cleaved by TPP (data not shown).

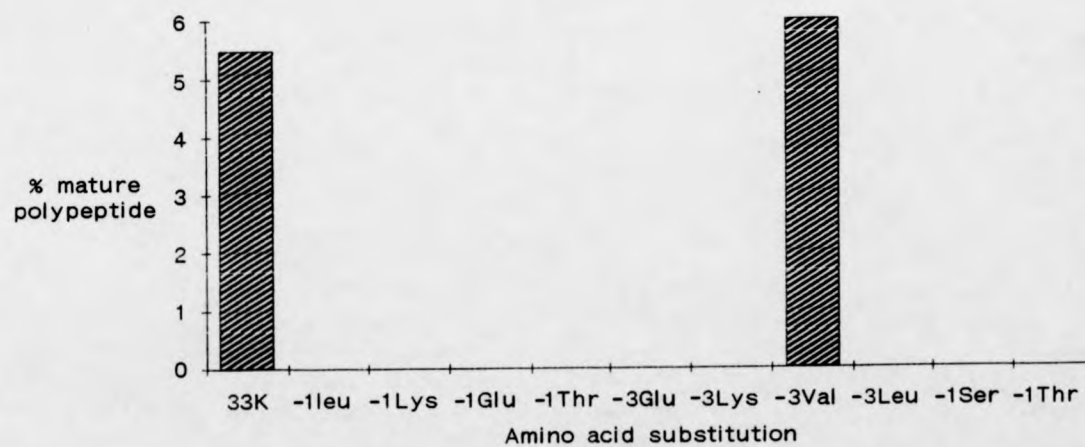
Panel b

The mature bands present in the above processing experiment were quantitated by laser densitometry.

panel a



panel b



tolerate a valine at the -3 position but none of the other substitutions tested. It is interesting to note that valine, a small hydrophobic amino acid like alanine is one of the deviation seen from the -3Ala X -1Ala conserved sequence, section 1:2:c and Jansen *et al.*, (1987). The recognition by TPP of pre-33K is very inefficient in the above in vitro processing assays, and it may be that TPP does process these other mutations but at an efficiency that can not be detected by the use of SDS-PAGE and fluorography.

Section 3:8:c Import Of -1 and -3 Mutant Proteins Into Chloroplasts

Section 3:8:c:i Fractionation Studies

In the previous section it was shown that TPP fails to process -3Leu, -3Lys, -3Glu, -1Leu, -1Lys, -1Glu, -1Lys, -1Thr, -1Ser and -1Gly but does process -3Val to a mature size protein as judged by SDS-PAGE analysis followed by fluorography. This section will consider the fate of the ten mutants generated above when they are imported into isolated chloroplasts.

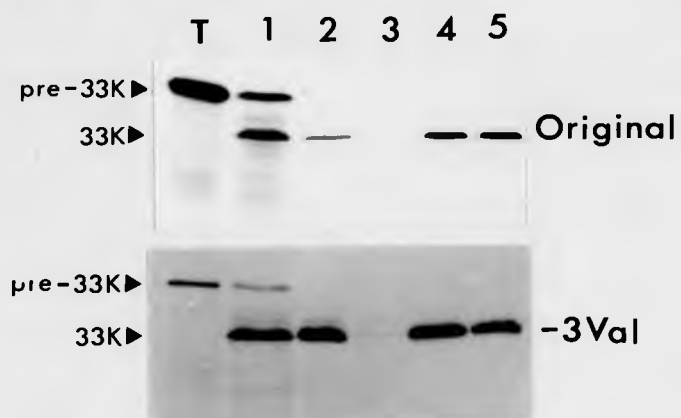
Wild type and mutant pre-33K's were incubated with isolated chloroplasts, and the localization of the imported polypeptides was determined by protease and fractionation treatments of the chloroplasts (section 2:22). Figure 35 shows that all of the mutants are targeted into the thylakoid lumen with essentially equal efficiency; in each case, the imported protein is located in the thylakoid fraction and is resistant to added protease indicating a location within the luminal phase. The mutations do not, therefore, alter the membrane translocation properties of

Figure 35 Import of pre-33K mutant proteins into isolated chloroplasts

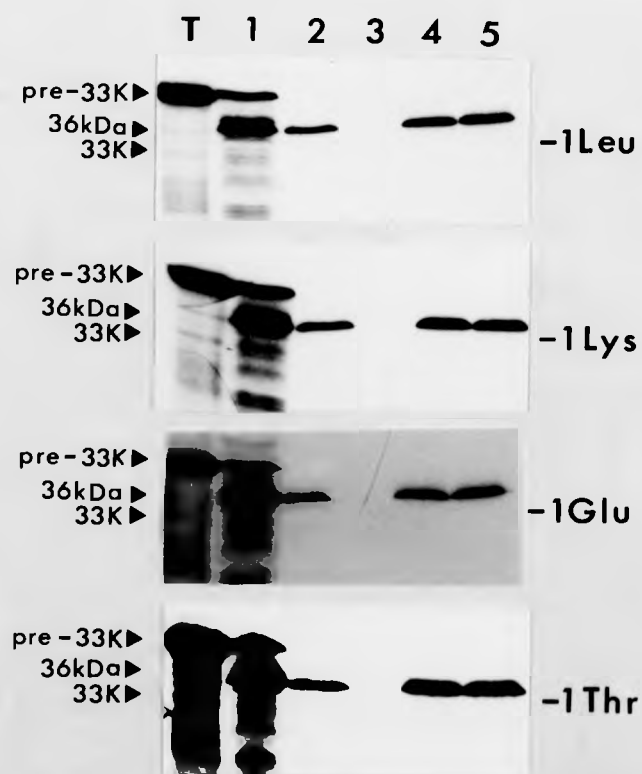
Non-mutated and mutated pre-33Ks were synthesised by in vitro transcription-translation as described under "Materials and Methods" (sections 2:23 and 2:24). Each translation (lane T) was incubated with intact chloroplasts at 25°C for 20mins under illumination after which the chloroplasts were either washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol and then lysed in 10mM Tris pH8.0 (lane 1) or were washed and treated with 2.5µl of 5mg/ml of trypsin for 40mins on ice. After incubation, these chloroplasts were washed with 50mM HEPES-KOH pH8.0, 330mM sorbitol, containing 2.5µl of 5mg/ml of trypsin inhibitor, and either analysed directly (lane 2) or after lysis in 10mM Tris pH7.6 and fractionation into stromal (lane 3) and thylakoidal fractions. The thylakoidal fraction was either analysed directly (lane 4) or was treated with 2µl of 1mg/ml of proteinase K for 30mins on ice before being washed in 10mM Tris pH7.6, containing 50µl of 7mg/ml PMSF and resuspended in 10mM Tris pH7.6 (lane 5). All samples were added to one volume of 2x sample buffer and boiled for 2mins, and were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as in figure 17.

Class 1

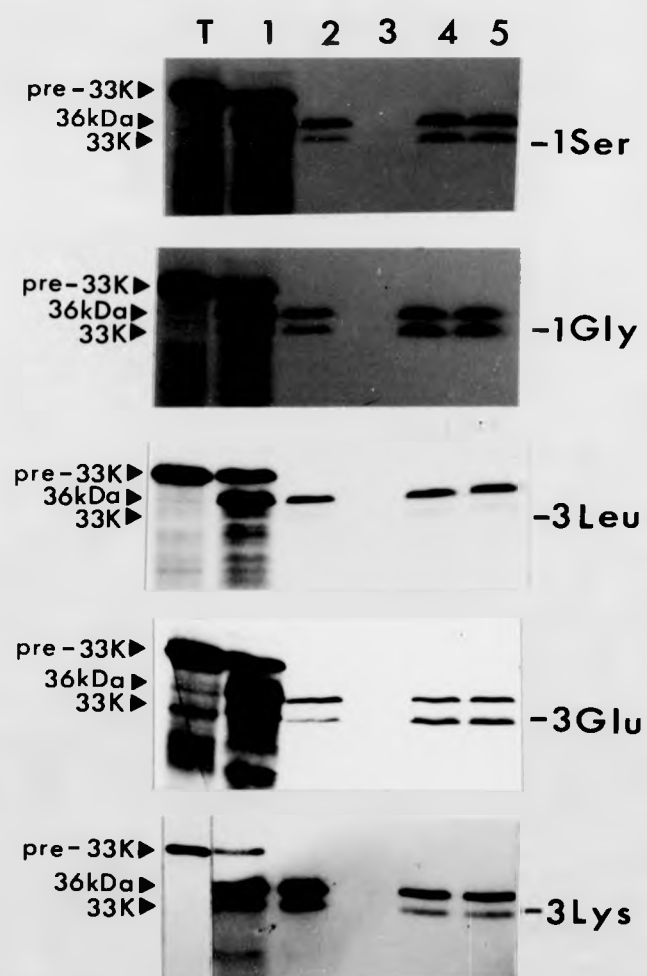
205



Class 2



Class 3



the precursors in any detectable manner. However, an analysis of the sizes of the imported proteins indicates that the substitutions can be divided into three classes based on their effects on maturation by TPP:-

Class 1. Those that can be imported into isolated chloroplasts and are targeted to the thylakoid lumen where they are processed by the TPP to a mature sized protein. There was only one member to this class: the -3Val mutant. This mutant was imported into and localized within the chloroplast in a manner identical to that of wild-type pre-33K. It seems from the processing and import data that the substitution of a valine residue at the -3 site of the pre-33K not only does not affect processing by isolated TPP but also has no affect on import into and localization within the chloroplast.

Class 2. The second class are those mutants that are imported into the chloroplast and are targeted to the thylakoid lumen but are not processed to a mature sized protein. Instead they are processed only to a 36kDa protein, which is intermediate in size between the stromal intermediate and the mature protein. The -1Leu, -1Lys, -1Glu and -1Thr mutations fall in this second class as seen from figure 35. None of these mutations were processed by TPP in the in vitro processing assay carried out in section 3:5:b. From the processing and import data it appears that the TPP cleavage site was completely blocked by the substitution of leucine, lysine, glutamate or threonine at the -1 position and that a second site exists within the pre-sequence for cleavage. It is unclear if this second cleavage event was a result of TPP activity or another protease yet to be identified. This possibility was examined further by applying an algorithm

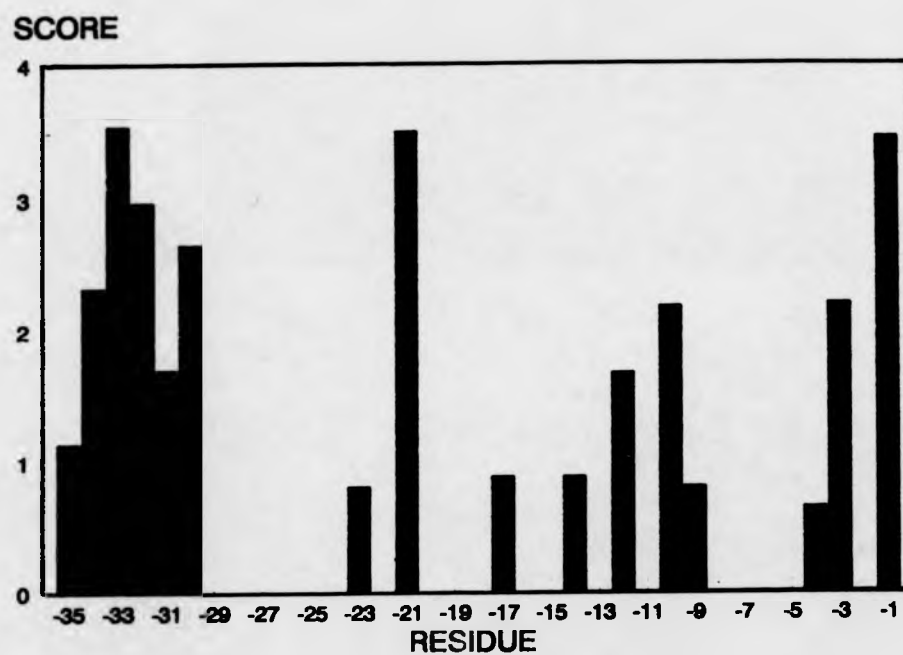
developed by Von Heijne, (1986) to the TTD of the 33K pre-sequence (analysis kindly carried out by Dr. C. Howe (Cambridge)). This algorithm was developed for predicting processing sites within prokaryotic signal sequences, and, given the similarities in reaction specificity exhibited by TPP and LEP, might also be expected to predict sites of cleavage by TPP. Figure 36 shows that within the carboxyl-terminal 35 residues of the 33K pre-sequence (the approximate extent of the TTD) several potential cleavage sites are predicted. The algorithm correctly predicts the bona fide cleavage site, since a high score is given for cleavage after the -1 alanine. However, a high score is also given for cleavage after the -21 residue (also alanine) and this would be consistent with a product size of 36kDa. These data therefore strengthen the proposal that the 36kDa form is generated by TPP.

The fact that TPP does not recognise this predicted second TPP cleavage site in in vitro processing experiments may support the notion of a second protease, or may simply represent differences in the ability of TPP to recognise the pre-33K in in vitro processing assays and chloroplast imports. It should be noted that TPP only recognises pre-33K inefficiently when compared to other precursors such as those of the wheat 23kDa OEC and silene plastocyanin, so this second predicted cleavage site may be recognised at a very low efficiency in the in vitro processing assays.

Class 3. The third and final class of mutants are those that are imported into the chloroplast and are targeted to the thylakoid lumen where they are processed both to the mature size protein and to the 36kDa intermediate seen in class 2. The mutants in

Figure 36 Prediction of signal peptidase cleavage sites within
the 33K pre-sequence

Potential cleavage sites were predicted by subjecting the carboxyl-terminal 35 residues of the 33K pre-sequence to an algorithm by von Heijne, (1986) for predicting cleavage sites within prokaryotic signal peptides. Only scores greater than 0 have been plotted; higher scores represent a greater probability of cleavage taking place immediately after the residue.



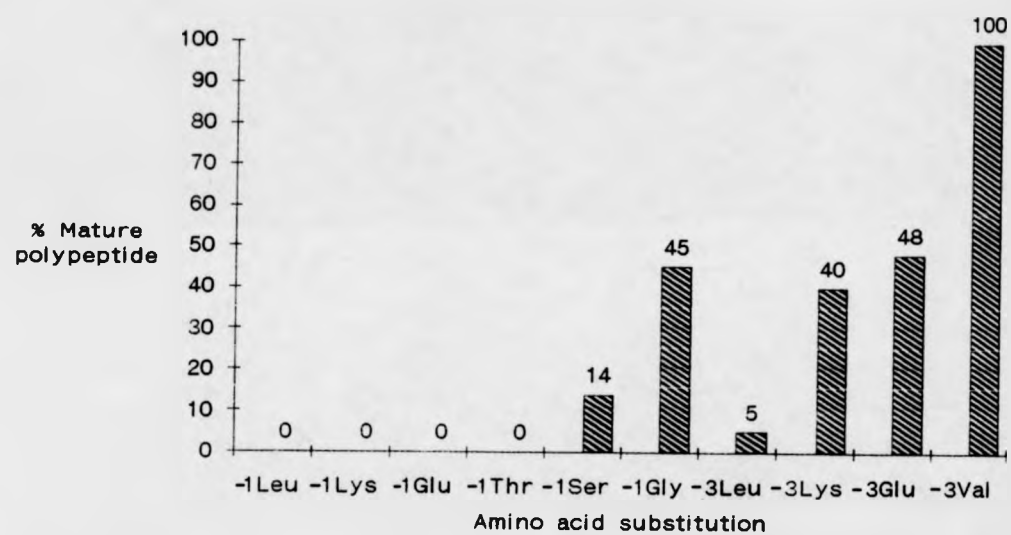
this class are -3Leu, -3Glu, -3Lys, -1Ser and -1Gly. The ratios of 36kDa intermediate and mature protein vary with each mutation in this class as seen in figure 35. The variation in the intermediate to mature ratio most likely reflects the efficiency with which TPP can recognise the mutant precursors at the -1 TPP cleavage sites. Figure 37 presents this ratio as a % of mature protein, these figures were based on the intensities of the intermediate and mature forms seen in the protease treated thylakoidal fraction (figure 35, lane 5) and were determined by laser densitometry. From these figures it is clear that processing by TPP is more tolerant of amino acid substitutions at the -3 site than at the -1 site. At the -1 site TPP is able to recognise the -1 cleavage site when glycine or serine are present but the -1 site is not recognised when leucine, lysine, glutamate or threonine are present at the -1 site. Cleavage at the correct site was not completely blocked in any of the -3 mutants assayed using in vitro chloroplast imports; cleavage occurred when leucine, lysine or glutamate were present at the -3 site in contrast to the result seen when these amino acids were present at the -1 site. From the ranking of % mature protein produced in the -3 mutants, it would appear that the charge of substituted amino acid is less important than surface area occupied.

Section 3:8:c:ii The Kinetics of Import Into Chloroplasts of the 36kDa Intermediate and Mature 33K Polypeptide

The data shown in figures 35 and 37 are useful for comparing the effects on cleavage of various substitutions at the -3 and -1 positions, and allow different side chains to be ranked to some

Figure 37 The % mature polypeptide present when mutant pre-33Ks
were imported into isolated chloroplasts

The intensity of the 36kDa and mature bands present in the protease treated thylakoid fraction (lane 5 figure 35) of the mutated pre-33Ks were quantitated by laser densitometry. These intensities were expressed as a % of mature polypeptide present.



extent, according to their effects on TPP. However, the data do not necessarily give an accurate indication of the extent to which a given substitution inhibits cleavage by TPP at the correct site, especially in the class 3 mutants. This is because all of the data shown in figures 35 and 37 were generated from chloroplast import incubations of 20mins, a period which was chosen so that multiple incubations could be carried out with little sampling error. Figure 38 shows the results of a time course analysis of the maturation of the -1Gly and -1Leu mutants, which was carried out by taking time-point samples during a import incubation and rapidly processing these samples (by omitting the protease treatment and the fractionation procedure). The data for the -1Gly mutant clearly shows that at early time points, imported -1Gly is present almost entirely as the 36kDa polypeptide, and that this form is gradually converted to 33K during the import incubations. The impression given in figure 37 for the -1Gly mutant thus grossly underestimates the extent to which this substitution inhibits cleavage at the correct site. Rather than cleaving at the correct (-1) and alternative (-21) sites at essentially equal rates it is clear that TPP displays a massive preference for the alternative site.

The kinetic pattern shown by the -1Gly mutant suggests that the 33K polypeptide is derived from the 36kDa polypeptide due to inefficient cleavage by TPP. Although the reduction in the 36kDa intermediate seen upon import of the -1Gly mutation into chloroplasts could also be explained if the intermediate was degraded by a non-specific protease in the thylakoids. This can be shown not to be the case when the time course uptake into chloroplasts of the -1Leu mutation (which is only processed to

extent, according to their effects on TPP. However, the data do not necessarily give an accurate indication of the extent to which a given substitution inhibits cleavage by TPP at the correct site, especially in the class 3 mutants. This is because all of the data shown in figures 35 and 37 were generated from chloroplast import incubations of 20mins, a period which was chosen so that multiple incubations could be carried out with little sampling error. Figure 38 shows the results of a time course analysis of the maturation of the -1Gly and -1Leu mutants, which was carried out by taking time-point samples during a import incubation and rapidly processing these samples (by omitting the protease treatment and the fractionation procedure). The data for the -1Gly mutant clearly shows that at early time points, imported -1Gly is present almost entirely as the 36kDa polypeptide, and that this form is gradually converted to 33K during the import incubations. The impression given in figure 37 for the -1Gly mutant thus grossly underestimates the extent to which this substitution inhibits cleavage at the correct site. Rather than cleaving at the correct (-1) and alternative (-21) sites at essentially equal rates it is clear that TPP displays a massive preference for the alternative site.

The kinetic pattern shown by the -1Gly mutant suggests that the 33K polypeptide is derived from the 36kDa polypeptide due to inefficient cleavage by TPP. Although the reduction in the 36kDa intermediate seen upon import of the -1Gly mutation into chloroplasts could also be explained if the intermediate was degraded by a non-specific protease in the thylakoids. This can be shown not to be the case when the time course uptake into chloroplasts of the -1Leu mutation (which is only processed to

Figure 38 Time course analysis of import of the -1Gly and -1Leu mutants into chloroplasts

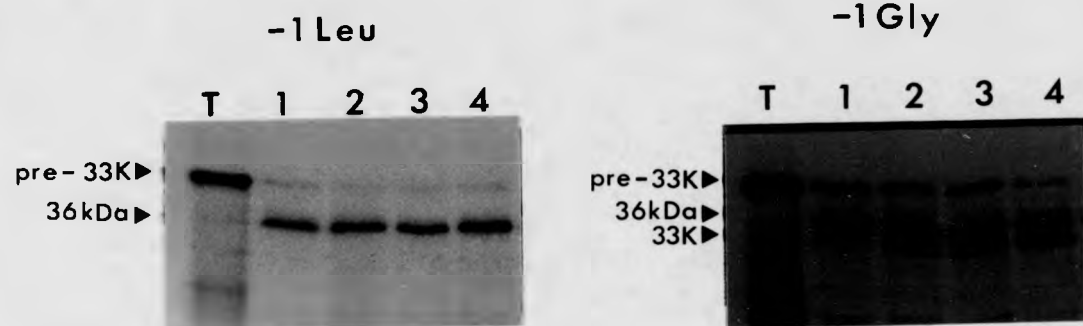
Panel a

The -1Gly and -1Leu mutants were synthesised by in vitro transcription-translation as described under "Materials and Methods" (sections 2:23 and 2:24). Each translation (lane T) was incubated with intact chloroplasts at 25°C for 2.5 (lane 1), 5 (lane 2), 10 (lane 3) and 20mins (lane 4) under illumination, after which the chloroplasts were washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol and then lysed in 10mM Tris pH8.0 and were added to one volume of 2X sample buffer and boiled for 2mins. All samples were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as in figure 17.

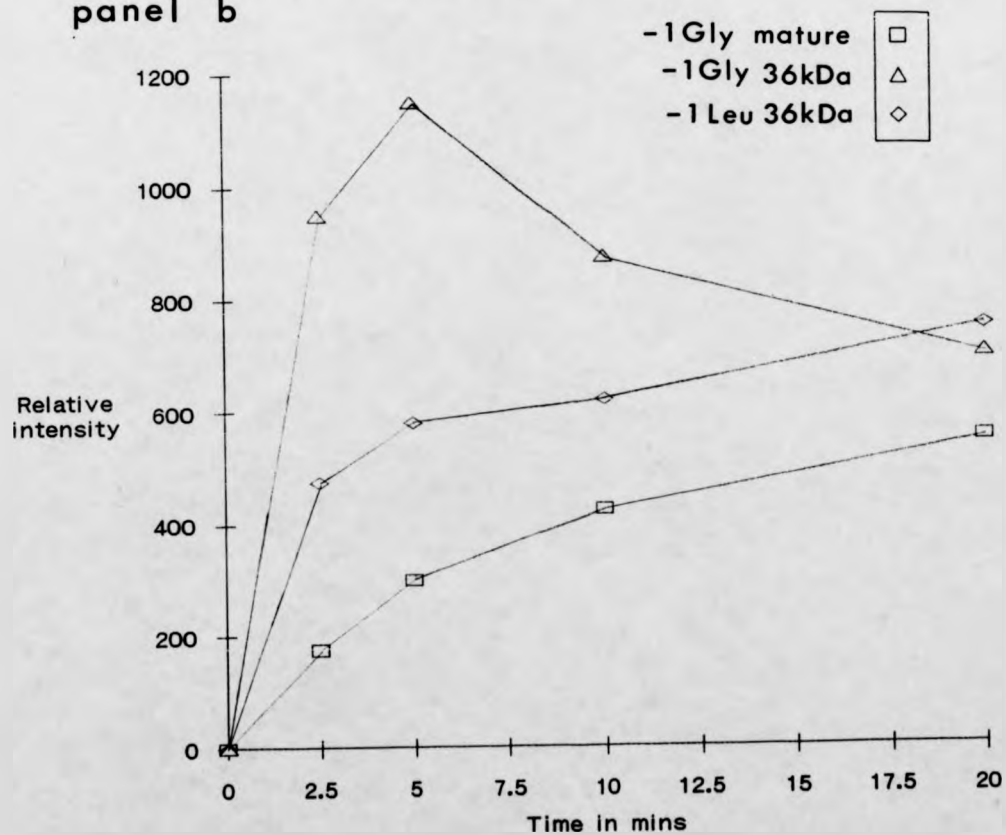
Panel b

The intensities of the 36kDa intermediate and 33K mature bands generated at the various time points were quantitated by laser densitometry. These relative intensities are plotted against time of incubation.

panel a



panel b



the 36kDa intermediate) is considered. In this time course the 36kDa intermediate accumulates over the period of the time course and does not show a decrease after a maxima as seen for the 36kDa intermediate of the -1Gly mutation, indicating that the 36kDa intermediate is stable. From this it would appear that in the case of class 3 mutants the stromal intermediate is efficiently processed by TPP to the 36kDa intermediate, this intermediate is then processed more slowly to the mature protein.

Section 3:8:c:iii Sonication Study Of The -1Gly Mutant Protein

The fractionation study shown in figure 35 clearly indicates that the 36kDa intermediate is located in the thylakoid lumen. However, it is not clear whether the 36kDa intermediate can associate with photosystem II (PSII) as does the mature protein. To address this question the -1Gly mutant protein was imported into chloroplasts and thylakoids were subsequently isolated, and sonicated (section 2:30), the data from such an experiment is in figure 39. The -1Gly mutation was chosen for this analysis because it had been previously shown to be processed to the 36kDa intermediate and the mature protein in approximately a 1:1 ratio. The use of this mutation removes any variations that might occur when comparing two sonication experiments. As can be seen by comparison of the pellet and supernatant with and without sonication, the mature protein is released to a greater extent than the 36kDa intermediate when the sample was sonicated (30% of the mature polypeptide as compared to 7% of the 36kDa intermediate). This demonstrates that the 36kDa intermediate does not associate with the thylakoid membrane in the same way as

Figure 39 Effects of sonication on the release of the 36kDa intermediate and 33K from thylakoids

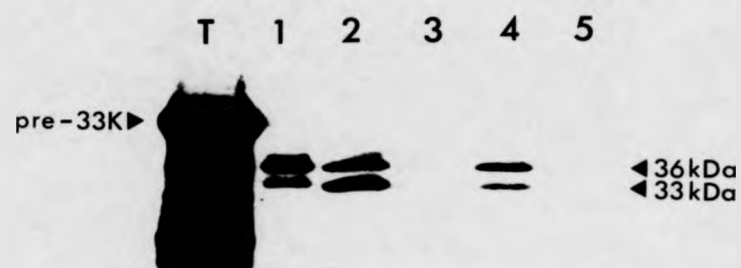
Panel a

The -1Gly mutant was synthesised by in vitro transcription-translation as described under "Materials and Methods" (sections 2:23 and 2:24). The translation mixture (lane T) was incubated with intact chloroplasts at 25°C for 20mins under illumination after which the chloroplasts were either washed in 50mM HEPES-KOH pH8.0, 330mM sorbitol and then lysed in 10mM Tris pH8.0 (lane 1) or were washed and lysed in 10mM Tris pH8.0. These lysed chloroplasts were sonicated at maximum amplitude (MSE) for 5 (lanes 2 and 3) or 20 seconds (lanes 4 and 5), after which the samples were centrifuged at 100000*g for 30mins to generate membrane (lanes 2 and 4) and supernatant (lanes 3 and 5) samples. All samples were added to one volume of 2X sample buffer and boiled for 2mins, and were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Notation as in figure 17.

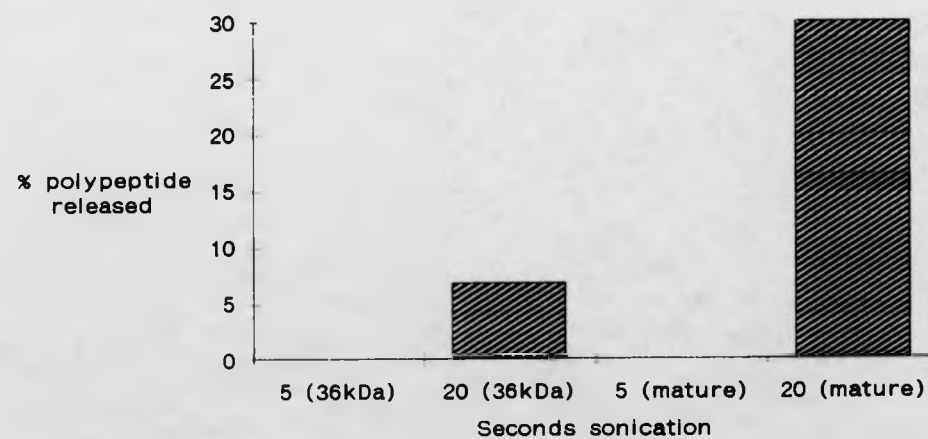
Panel b

The intensities of the 36kDa intermediate and 33K mature bands generated in the membrane and supernatant samples were quantitated by laser densitometry. These intensities are expressed as the % polypeptide released into the supernatant sample.

panel a



panel b



mature 33K; it may be that the remaining TTD present in the 36kDa intermediate is anchoring the intermediate in the membrane. Thus the removal of the TTD may be essential for the release of the mature protein from the thylakoid membrane into the thylakoid lumen. Support for this comes from the observation that when pre-23K and pre-33K are not processed by TPP in the lut-1 mutation of tomato plants they do not associate with PSII because they are embedded in the thylakoid membrane (section 1:3:b)(Chia et al., 1986). The mutagenesis work above and the lut-1 work have indicated that TPP cleavage at the -1 site is not required for targeting across the thylakoid membrane.

Section 3:8:d Expression Of -1 and -3 Mutant Proteins In Escherichia coli

When pre-33K was subcloned into the pKK233-2 expression vector to create pKK233-2-33 and used to transform E.coli mature size 33K protein was detected in the periplasmic space of the E.coli on induction with IPTG (Meadows et al., in press)(section 1:8:c). It would appear that the bacterial plasma membrane export machinery can recognise the pre-sequence of 33K, and target the pre-33K across the bacterial plasma membrane into the periplasmic space (section 1:8:c). The ten mutations created in section 3:5:a were subcloned into pKK233-233. When these were expressed in E.coli (section 2:28), a mature sized protein was produced for -1Leu, -1Ser, -3Leu, -3Lys, -3Glu, -1Gly and -3Val mutations, figure 40. However, with mutations -1Lys, -1Glu, and a protein slightly larger than mature size was produced, figure 40 (it was not possible to express the -1Thr mutation in E.coli). From the

Figure 40 Expression of the pre-33K mutant proteins in E.coli

The pre-33K mutant proteins were subcloned into the pK233-2-33 expression vector as described in Materials and Methods section 2:28. The resulting mutant vectors were grown in 5ml of LB containing 5 μ l of 50 μ g/ml ampicillin overnight at 37°C with agitation. 50 μ l of this overnight culture was used to infect a fresh 5ml of LB containing 5 μ l of 50 μ g/ml ampicillin. This was grown at 37°C with agitation until the O.D.₆₀₀ reached 0.2 units. The expression vector was induced by the addition of IPTG to a final concentration of 1mM. This induced culture was grown for a further 3hrs at 37°C with agitation. After this incubation 1ml of the culture was pelleted by centrifugation in a microfuge for 10 minutes. The pelleted cells were resuspended in 50 μ l of LB and subjected to SDS-PAGE (section 2:21). The proteins were transferred onto nitrocellulose by the semi-dry blot method (section 2:27) and were probed using polyclonal antibodies to the pre-33K (kindly donated by J.Musgrove (Warwick)) by the Biotin-streptavidin / 4 chloro-1-naphthol method Materials and Methods section 2:27. Lanes 1-10: -1Leu, -1Lys, -1Glu, -1Thr, -3Glu, -3Lys, -3Val, -3Leu, -1Ser, -1Gly, Lane 11 pK233-2-33, Lane 12 pK233-2, Lane 13 Polypeptides of the OEC (donated by H. James (Warwick)). The -1Thr mutant did not express in this or subsequent experiments for unknown reasons.

1 2 3 4 5 6 7 8 9 10 11 12 13



◀33K

above E.coli expression work it would appear that none of the substitutions made at the -3 position affected processing by LEP at the -1 cleavage site as judged by SDS-PAGE and Western blot analysis. The substitution of a leucine, glycine, threonine or serine residue at the -1 site also did not affect cleavage at the -1 site by LEP; however, the substitution of a lysine or glutamate residue at the -1 position blocks cleavage by LEP at the -1 site. In these blocked precursors an alternative cleavage site close to the -1 site can be recognised. It is not clear if this second cleavage site results from the cleavage by LEP or another protease, but computer analysis has identified a number of sites within the precursor that could conceivably be recognised by LEP, computer analysis kindly carried out by Dr.C. Howe (Cambridge)(figure 36) using the von Heijne algorithm for the prediction of signal peptidase cleavage (von Heijne, 1986). The mature sized protein produced by the -1Leu mutation and the larger intermediate produced by the -1Lys mutation are both targeted to the periplasmic space as is wild type 33K (data not shown). The fact that the larger intermediate generated when the -1 cleavage site was blocked could be located to the periplasmic space suggests that this alternative cleavage was the result of LEP activity and not a protease located in the cytoplasm, although it does not prove this point. Size analysis suggests that when the -1 cleavage site was blocked the intermediate generated by LEP cleaving at an alternative site was not the same as the 36kDa intermediate generated when TPP cleaves at an alternative site.

Section 3:8:e Summary Of Oligonucleotide Mutagenesis Analysis
Of TPP Cleavage Site.

Section 3:8:e:i Chloroplast Import Analysis

None of the residues substituted at the -1 or -3 positions of the 33K pre-sequence affected translocation across either the chloroplast envelope or thylakoid membrane; the substitutions could, however, be characterised by their maturation by TPP.

Of the mutant proteins created with mutations at the -3 site only -3Val was processed solely to the mature 33K indicating that valine acts as an efficient substrate for TPP cleavage at the -1 site; this result is in complete agreement with the published pre-sequence data where a valine residue is seen at the -3 position in the 16kDa OEC polypeptide of spinach. Sequence analysis has also shown that a serine is present at the -3 position of the CF₀II polypeptide of spinach, which suggests that serine can act as an efficient substrate for TPP cleavage at the -1 site.

When a -3Leu residue was substituted the cleavage at the -1 site was markedly inhibited and an alternative cleavage site was revealed resulting in the formation of a 36kDa intermediate. The leucine side chain contains an additional -CH₂- unit to that of the valine side chain and it would appear that the larger surface area of the leucine side chain at the -3 position causes the dramatic reduction in TPP cleavage at the -1 site. The -3leu mutation falls into chloroplast import class 3 as defined in section 3:5:c, two other mutations (-3Lys and -3Glu) created at the -3 position fall into this class, both of these mutations process more of the thylakoid lumenal targeted protein to the

mature from than the -3Leu mutation. This indicates that TPP cleavage was less effected by the basic (lysine side-chain $(\text{CH}_2)_4\text{NH}_3^+$) and acidic (glutamate side-chain $(\text{CH}_2)_2\text{CO}_2$) side chains of the -3Lys and -3Glu mutants than the branched side chain of the leucine residues $\text{CH}_2\text{CH}(\text{CH}_3)_2$. It would appear that the surface area occupied by side chains at the -3 site is more critical to the blockage of TPP cleavage at the -1 site than the charge of the side chain.

At the -1 position the introduction of a serine or glycine residue, whose side chains occupy a similar surface area to that of alanine, caused a marked inhibition of TPP cleavage at the -1 site. It would appear that even slight increases or decreases in the surface area of the side chain introduced at the -1 site reduces the ability of TPP to cleave at the -1 site. The introduction of a -1Leu, -1Thr, -1Glu or -1Lys blocks TPP cleavage completely. Of the residues tested, the -1 site specifically require alanine at this position for efficient cleavage to take place. Table 1 summarises the inhibition of TPP cleavage at the -1 site with respect to residues introduces and site.

When the -1 cleavage site was blocked an alternative cleavage site was recognised by TPP, this alternative site could also be predicted using an algorithm based on the -3, -1 rule (the computer predicted and experimental cleavage sites have not been proved to be the same). It would then appear that the presence of residues of a similar size to alanine at the -3 and -1 positions forms a key requirement for TPP cleavage. It would be unlikely that this alternative cleavage site would have the same secondary structure context as the -1 cleavage site, suggesting

that TPP may require a simple recognition sequence for cleavage to take place.

Section 3:8:e:ii Analysis of mutant expression in
Escherichia coli

The -3Val, -3Glu, -3Leu, -1Ser, -1Gly, -1Leu and -3Lys mutations were all recognised by LEP when expressed in E.coli and processed at the -1 cleavage site, as judged by size analysis. The -1Lys and -1Glu mutations were not processed at their -1 cleavage sites when expressed in E.coli. This study suggests that LEP is more restrictive of amino acid substitutions at the -1 site than the -3 site of the 33K pre-sequence; a similar situation was seen with TPP. TPP was however, more restrictive with respect to amino acid that could be substituted at the -3 and -1 position than LEP (cf. panels a and b figure 41). This point is clearly illustrated in the case of the -1Leu mutation, TPP failed to recognise the -1 cleavage site when the mutant precursor was taken up by isolated chloroplasts whereas, LEP did recognise the -1 cleavage site when expressed in E.coli. This demonstrates that LEP and TPP do not possess identical reaction specificities; of the amino acids analysed TPP will only tolerate alanine or valine at the -3 position and alanine at the -1 position whereas, LEP will tolerate alanine, glutamate, lysine, valine and leucine at the -3 position and alanine, leucine, glycine and serine at the -1 position (figure 31).

Section 3:8:f Comparisons with LEP and SP reaction specificities

Halpin et al., (1989) have shown a similarity in the reaction specificities of the processing of periplasmic, thylakoid lumenal, and probably E.R. targeting signals (section 1:8:c). Both LEP and TPP will process some eukaryotic and bacterial leader peptides and TTDs; it should be noted that Halpin et al., (1989) chose to test pre-sequence with the Ala X Ala motif. LEP and eukaryotic signal peptidase (SP) have also been shown to have similar reaction specificities by their ability to process each others leader peptides. Von Heijne et al (1985) have used sequence analysis to identify a conserved -3 X -1 motif immediately before the cleavage sites of LEP, SP or TPP (section 1:8:a). These processing experiments and sequence analysis studies have all suggested that LEP, SP and TPP are related. Folz et al., (1988), and Fikes et al., (1990) have used site directed mutagenesis to study the substrate specificity of LEP and SP respectively. A comparison of results of the above studies with those derived from the site directed mutagenesis study of the pre-33K would enable conclusions to be drawn about the similarities of substrate specificity between LEP, SP and TPP Table 1 summarises the data presented above and the results of Folz et al., (1988), and Fikes et al., (1990) (also see figures 41 and 42).

Alanine, serine and glycine when located at the -1 residue can all be tolerated by LEP, SP and TPP, although with varying processing efficiencies: LEP was essentially unaffected by either of these residues whereas, SP showed a reduced processing

Table 1 Summary of reaction specificities of TPP, LEP and SP based on the data presented above and in Fikes et al., (1990) and Folz et al., (1988)

	pre-MBP	pro(detapro)apoAII	pre-33K	pre-33K
	LEP	SP	TPP	LEP
	-1 SITE			
Processing to mature	Ala, Gly, Ser, Cys (100%)	Ala, Cys (100%)	Ala (100%)	Ala, Ser, Leu, Gly (100%)
0%	Thr (25%)	Gly (53)	Gly (45)	Lys, Glu (0%)
	Asp, Val, Asn, His, Ile, Arg, Leu, Tyr, Phe (0%)	Asn, His, Arg, Asp, Ile, Leu, Tyr, Val (0%)	Ser (14%) Thr, Glu, Lys, Leu (0%)	
	-3 SITE			
Processing to mature	Ala, Gly, Ser, Leu, Thr, Val, Cys, Ile (100%)	-	Ala, Val (100%)	Leu, Lys, Glu, Val (100%)
0%	Pro (90%)	-	Glu (48%) Lys (40%)	
	Asn (25%) Asp (10%) Arg, His, Phe, Tyr (7%)	-	Leu (5%)	

efficiency with -1Ser and -1Gly, and TPPs processing efficiency as markedly inhibited by -1Ser and -1Gly. When the efficiency of cleavage of SP and LEP for the -1Ser and -1Gly substitutions was compared to that of TPP cleavage, TPP cleavage was essentially blocked. A clear difference between the reaction specificities of TPP and that of LEP and SP at the -1 site was seen with respect to the -1Thr mutation. When threonine was located at the -1 site of the TTD it effectively blocked processing by TPP, whereas a threonine residue located at the -1 site of the eukaryotic or prokaryotic signal peptide was recognised by SP and LEP respectfully. The reaction specificities of LEP and TPP were also different with respect to the -3 position. When a -3Leu was introduced into the chloroplast pre-sequence TPP cleavage was essentially blocked, whereas a leucine residue at the -3 position of the prokaryotic signal peptide had no effect on LEP cleavage. Unfortunately the site directed study of Folz *et al.*, (1988) was only carried out at the -1 site so it is not possible to compare the reaction specificities of LEP and TPP with those of SP at the -3 site.

The comparison of the reaction specificities of LEP, SP and TPP reveals that although they all have similar reaction specificities they are not identical.

Figure 41 Residues that are tolerated by TPP and LEP at either the -3 or -1 position of the 33K pre-sequence

Panel a

The -3Ala X -1Ala conserved motif of hydrophobic targeting signals is shown. The residues below indicate those that are tolerated by TPP when substituted in the 33K pre-sequence. The bracketed Ser indicates that pre-sequence analysis suggests that a serine residue can also be tolerated at the -1 position (section 1:6:c).

Panel b

The -3Ala X -1Ala conserved motif of hydrophobic targeting signals is shown. The residues below indicate those that are tolerated by LEP when substituted in the 33K pre-sequence and expressed in E.coli.

TPP

-3Ala X -1Ala
 Val
 (Ser)

Panel a

LEP

-3Ala X -1Ala
 Glu Leu
 Lys Ser
 Val Gly
 Leu

Panel b

Figure 42 Residues that are tolerated by SP at the -1 position of pro (deta pro) apoAII pre-sequence or by LEP at the -3 and -1 positions of the MBP pre-sequence

Panel a

The -3Ala X -1Ala conserved motif of hydrophobic targeting signals is shown. The residues below indicate those that are tolerated by SP when substituted in the -1 position of the pro (deta pro) apoAII pre-sequence.

Panel b

The -3Ala X -1Ala conserved motif of hydrophobic targeting signals is shown. The residues below indicate those that are tolerated by LEP when substituted in the MBP pre-sequence.

SP

-3Ala X -1Ala
 n d Cys

Panel a

LEP

-3Ala X -1Ala
 Gly Gly
 Ser Ser
 Leu Cys
 Thr
 Val
 Cys
 Ile
 Pro

Panel b

SECTION IV - OVERALL SUMMARY

The work I have undertaken during this project has resulted in the isolation of the cDNA encoding the pre-33K. The 5' end of this cDNA has been sequenced and the deduced amino acid sequence shares a number of common features with the now growing number of published thylakoid lumen targeted sequences. Pre-33K has been used to confirm the initial observation of Hageman et al., (1986); Smeekens et al., (1986) and Chia et al., (1986) that thylakoid lumen targeted proteins are synthesised with a bipartite pre-sequence. The ETD of these precursors targets them across the chloroplast envelope where the ETD is removed by SPP, the TTD then targets this stromal intermediate across the thylakoid membrane where the TTD is removed by TPP.

The study into effect of IAA modification on the thylakoid lumen targeted polypeptide, pre-23K demonstrated for the first time that targeting to the thylakoid lumen does not require the generation of a stromal intermediate as suggested in the "Two step model". The precursor form of 23K can target across the thylakoid membrane both in chloroplast uptake experiments (in organello) and in thylakoid vesicle uptake experiments (in vitro). This study also demonstrated clearly for the first time that the TTD can act as an internal targeting signal and does not require an N-terminal location as previously assumed. The IAA modification of pre-33K blocked import across the thylakoid membrane; this inability to target to the thylakoid lumen probably results from a failure of the TTD to interact correctly with thylakoid.

The reaction specificity of TPP has been defined for pre-33K

at the -3 and -1 positions for a number of amino acids by site directed mutagenesis. Halpin et al., (1989) suggested that the reaction specificity of TPP and LEP are similar. When the reaction specificities of TPP and LEP (Fikes et al., 1990) were compared it was clear that LEP and TPP had similar but not identical reaction specificities. A number of substituted residues blocked cleavage at the -1 site, it was shown that the surface area occupied by the side chain was the critical factor in determining TPP cleavage at the -1 site. When the -1 site was blocked TPP cleaved at a second site, the -21 site giving rise to a 36kDa intermediate. This intermediate although targeted across the thylakoid membrane was not associated with the membrane in a manner identical to that of 33K.

The work presented in this thesis has been included in the content of the following publication.

ATP-dependent import of a luminal protein by isolated thylakoid vesicles. Kirwin, P.M., Meadows, J.W., Shackleton, J.B., Musgrove, J.E., Elderfield, P.D., Mould, R., Hay, N.A., Robinson, C. (1989) EMBO J. 8, 2251-2255.

Transport and processing of cytoplasmically synthesised chloroplast proteins. Mould, R., Elderfield, P., Shackleton, J.B., Robinson, C. (1989) Proceedings of the International Congress on Photosynthesis.

Transport of proteins across the thylakoid membrane. Shackleton, J.B., Mould, R., Robinson, C. (1990) Proceedings of the NATO FEBS

Meeting on Plant Molecular Biology, Elmau, FRG.

Transport of proteins into chloroplasts. Meadows, J., Shackleton, J.B., Bassham, D., Mould, R., Hulford, A., Robinson, C. (1991) SEB Seminar Series (in press)

Transport of proteins into chloroplasts. The thylakoidal processing peptidase is a signal-type peptidase with stringent substrate requirements at -3 and -1 positions. Shackleton, J.B., Robinson, C. The Journal of Biological Chemistry 266, 12152-12156 (1991).

Transport of proteins into chloroplasts. Requirements for the efficient import of two luminal oxygen-evolving proteins into isolated thylakoids. Mould, R., Shackleton, J.B., Robinson, C. (submitted to) The Journal of Biological Chemistry.

Efficient light driven import into isolated thylakoids of two luminal oxygen-evolving proteins, one of which is transported as either the precursor or intermediate form. Shackleton, J.B., Mould, R., Robinson, C. (1991) The Journal of Biological Chemistry (in press).

Protein targeting to chloroplasts. Mould, R., Shackleton, J.B., Robinson, C. (1991) Plant Genetic Engineering. Volume 4. (Miflin, B.J., ED. in press).

Section	<u>References</u>
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Akiyama, Y. & Ito, K., Topology analysis of the SecY protein, an integral membrane protein involved in protein export in E.coli. EMBO J. 6, 3465-3470 (1987).

Anderson, C.W., Monitoring cloned gene expression. Methods in Enzymology 101, 635-644 (1983).

Andersson, B., Sayre, R.T. & Bogorad, L., Transbilayer organisation of photosystem II proteins with speacial emphasis on the 32kDa Q_b-binding protein. Chemica Scripta 27B, 194-200 (1987).

Aron, D.I., Copper enzymes in isolated chloroplasts. Plant Physiol., 24, 1-15 (1949).

Baker, E.P. & Randall, L.L., The requirements for energy during export of beta-lactamase in E.coli is fulfilled by the total protonmotive force. EMBO J. 3, 895-900 (1984).

Bassford, P., Beckwith, J., Ito, K., Kumamoto, C., Mizushima, S., Oliver, D., Randall, L., Silhavy, T., Tai, P.C. & Wickner, B., The primary pathway of protein export in E.coli. Cell 65, 367-368 (1991)

Benson, S.A., Hall, M.A. & Silhavy, T.J., Genetic analysis of protein export in Escherichia cloi K12. Ann. Rev. Biochem. 54, 101-134 (1985).

De Boer, D., Cremers, F., Teertstra, R., Smits, L., Hille, J., Smeekens, S., & Weisbeek, P., In vivo import of plastocyanin and a fusion protein

into developmentally different plastids of transgenic plants. EMBO J. 7, 2631-2635 (1988).

Boulter, M., Ellis, R.J. & Yarwood, A., Chloroplast protein; synthesis and transport. Biol. Rev., 47, 113-175 (1972).

Boutry, M., Nagy, F., Poulsen, C., Aoyage, K. & Chua, N.H., Targeting of bacterial chlorophenicol acetyltransferase to mitochondria in transgenic plants. Nature 328, 340-342 (1987).

Boyle, S.A., Hemmingsen, S.M. & Dennis, D.T., Uptake and processing of the precursor to the small subunit of ribulose 1,5-bisphosphate carboxylase by leucoplasts from the endosperm of developing castor oil seeds. Plant Physiol. 81, 817-822 (1986).

Briggs, M.A., Cornell, D.G., Dluhy, R.A. & Gierasch, L.M. Conformations of signal peptides induced by lipids suggest initial steps in protein export. Science 233, 206-208 (1986).

Buvinger, W.E., Michel, H. & Bennett, J., A truncated analog of a pre-light-harvesting chlorophyll a/b protein II transit peptide inhibits protein import into chloroplasts. J. Biol. Chem. 264, 1195-1202 (1989).

Cashmore, A.R., in Genetic engineering of plants - an agriculture perspective (Kosuge, T., Meredith, C.P. & Hollaender, A., eds.) Basic life sciences vol.26. Plenum Press, New York, London, 55-67 (1983).

Chen, W.J. & Douglas, M.G., Phosphodiester bond cleavage outside

mitochondria is required for the completion of protein import into the mitochondrial matrix. Cell 49, 651-658 (1987).

Chia, C.P. & Arntzen, C.J., Evidence for two-step processing of nuclear-encoded chloroplast proteins during assembly. J. Cell. Biol. 103, 725-731 (1986).

Cline, K., Werner-Washburne, M., Andrews, J. & Keegstra, K., Thermolysin is a suitable protease for probing the surface of intact pea chloroplasts. Plant Physiol. 75, 675-678 (1984).

Cline, K., Werner-Washburne, M., Lubben, T.H. & Keegstra, K., Precursors to two nuclear-encoded chloroplast proteins bind to the outer envelope membrane before being imported into chloroplasts. J. Biol. Chem. 260, 3691-3696 (1985).

Cline, K., Fulsom, D.R. & Viitanen, P.V., An imported thylakoid protein accumulates in the stroma when insertion into thylakoids is inhibited. J. Biol. Chem. 264, 14225-14232 (1989).

Cornwell, K.L. & Keegstra, K., Evidence that a chloroplast surface protein is associated with a specific binding site for the precursor to the small subunit of ribulose 1,5-bisphosphate carboxylase. Plant Physiol. 85, 780-785 (1987).

Date, T. & Wickner, W., Isolation of the E.coli leader peptidase gene and the effects of leader peptidase overproduction in vivo. Proc. Nat. Acad. Sci. USA 78, 6106-6110 (1981).

Dennis, D.T., Energy transduction in the chloroplasts. The biochemistry of energy utilization in plants. Blackie P. 43-54(1987).

Dietrich, A., Souciet, G. & Weil, J.H., In vitro synthesis of bean (Phaseolus vulgaris) chloroplastic and cytoplasmic leucyl-tRNA synthetases. J. Biol. Chem. 262, 4248-4251 (1987).

Douce, R. & Joyard, J., Biochemistry and function in the outer membrane of the chloroplast envelope. Annu. Rev. Cell Biol. 6, 173-216 (1990).

Eilers, M., Oppliger, W. & Schatz, G., Both ATP and an energized inner membrane are required to import a purified precursor protein into mitochondria, EMBO J. 6, 1073-1077 (1987).

Eilers, M., & Schatz, G., Protein unfolding and the energetics of protein translocation across biological membranes. Cell 52, 481-483 (1988).

Ellis, R.J., The nuclear domination of chloroplast development. Sci. Prog. Oxf. 69, 129-142 (1984).

Ellis, R.J. & Robinson, C., Protein targeting. Adv. Bot. Res. 14, 1-23 (1987).

Ellis, R.J., Molecular chaperones: the plant connection. Science 250, 954-959 (1990).

Endo, T. & Schatz, G., Latent membrane perturbation activity of a mitochondrial precursor protein is exposed by unfolding. EMBO J. 7,

1153-1158 (1988).

Ferenci, T. & Silhavy, T.J., Sequence information required for protein translocation from the cytoplasm. *J. Bacteriology* 169, 5339-5342 (1987).

Fernaadez, M.M., Lamppa, G.K., Acyl carrier protein (ACP) import into chloroplasts does not require the phosphoantetheine: evidence for a chloroplast holo-ACP synthase. *The Plant Cell* 2, 195-206 (1990).

Fikes, J.D., Barkocy-Gallagher, G.A., Klapper, D.G. & Bassford, P.J., Jr., Maturation of E.coli maltose-binding protein by signal peptidase in vivo. *J. Biol. Chem.* 265, 3417-3423 (1990).

Flugge, U.I. & Benz, R., (1984) Pore-forming activity in the outer membrane of the chloroplast envelope. *Febs. Letts.* 169, 85-89 (1984).

Flugge, U.I. & Hinz, G., Energy dependence of protein translocation into chloroplasts. *Eur. J. Biochem.* 160, 563-570 (1986).

Flugge, U.I., Fischer, K., Gross, A., Sebald, W., Lottspeich, F. & Eckerskorn, C., The trio phosphate-3-phosphoglycerate translocator from spinach chloroplasts: nucleotide sequence of a full-length cDNA clone and import of the in vitro synthesized precursor proteins into chloroplasts. *EMBO J.* 8, 39-46 (1989).

Flugge, U.I., Import of proteins into chloroplasts. *J. Cell Sci.* 96, 351-354 (1990).

Folz, R.J., Nothwehr, S.F. & Gordon, J.I., Substrate specificity of eukaryotic signal peptidase. *J. Biol. Chem.* 263, 2070-2078 (1988).

Franzen, L.G., Rochaix, J.D. & von Heijne, G., Chloroplast transit peptides from green alga Chlamydomonas reinhardtii share features with both mitochondrial and higher plant chloroplast pre-sequences. *FEBS Letts.* 260, 165-168 (1990).

Freitag, H., Janes, M. & Neupert, W., Biosynthesis of mitochondrial porin and insertion into the outer mitochondrial membrane of Neurospora crassa. *Eur. J. Biochem.* 126, 197-202 (1982).

French, T. & Silhavy, T.J., Sequence information required for protein translocation from the cytoplasm. *J. Bacteriology* 169, 5339-5342 (1987).

Geller, B.L., Movva, N.R. & Wickner, W., Both ATP and the electrochemical potential are required for optimal assembly of pro-OmpA into E. coli inner membrane vesicles. *Proc. Nat. Acad. Sci. USA* 83, 4219-4222 (1988).

Grimm, B., Ish-shalom, D., Even, D., Glaczinski, Ottersbach, P., Ohad, I. & Kloppstech, K., The nuclear-coded chloroplast 22kDa heat-shock protein of Chlamydomonas. *Eur. J. Biochem.* 182, 539-546 (1989).

Grossman, A., Bartlett, S. & Chua, N.H., Energy-dependent uptake of cytoplasmically synthesised polypeptides by chloroplasts. *Nature* 285, 625-628 (1980).

Guiard, B., Structure, expression and regulation of a nuclear gene encoding a mitochondrial protein: the yeast L(+)-lactate cytochrome C oxidoreductase (cytochrome b_2). EMBO. J. 4, 3265-3272 (1985).

Hageman, J., Robinson, C., Smeekens, S. & Weisbeck, P., A thylakoid processing peptidase is required for complete maturation of the lumen protein plastocyanin. Nature 324, 567-569 (1986).

Hageman, J., Baecke, C., Ebskamp, M., Pilon, R., Smeekens, S., & Weisbeck, P., Protein import into and sorting inside the chloroplast are independent processes. The Plant Cell 2, 479-494 (1990).

Halpin, C., Elderfield, P.D., James, H.E., Zimmermann, R., Dunbar, B. & Robinson, C., The reaction specificities of the thylakoid processing peptidase and E.coli leader peptidase are identical. EMBO J. 8, 3917-3921 (1989).

Hansen, W., Garcia, P.D. & Walter, P., In vitro protein translocation across the yeast endoplasmic reticulum: ATP-dependent post translational translocation of the prepro-alpha-factor. Cell 45, 397-406 (1986).

Hartl, F.U., Ostermann, J., Guiard, B. & Neupert, W. Successive translocation into and out of the mitochondrial matrix: Targeting of proteins to the intermembrane space by a bipartite signal peptide. Cell 51, 1027-1037 (1987).

Hartl, F.U., Pfanner, N., Nickolson, D.W. & Neupert, W., Mitochondrial protein import. Biochimica et Biophysica Acta 988, 1-45 (1989).

Hawltischek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.U. & Neupert, W., Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein. *Cell* 53, 795-806 (1988).

Hinz, G. & Flugge, U.I., Phosphorylation of a 51kDa envelope membrane polypeptide involved in protein translocation into chloroplasts. *Eur. J. Biochem.* 175, 649-659 (1988).

Holmes, D.S. & Quigley, M., A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* 114, 193-197 (1981).

Huang, J., Hack, E., Thornburg, R.W. & Meyers, A.M. A yeast mitochondrial leader peptide functions in vivo as a dual targeting signal for both chloroplasts and mitochondria. *The Plant Cell* 2, 1249-1260 (1990).

Hurt, E.C., Soltanifar, N., Goldschmidt-Clermont, M., Roachaix, J.D. & Schatz, G., The cleavable pre-sequence of an imported chloroplast protein directs polypeptides into yeast mitochondria. *EMBO J.* 5, 1343-1350 (1986).

James, H.E., Bartling, D., Musgrove, J.E., Kirwin, P.M., Herrmann, R.G. & Robinson, C., Transport of proteins into chloroplasts: Import and maturation of precursors to the 33, 23 and 16kDa proteins of the photosynthetic oxygen-evolving complex. *J. Biol. Chem.* 264, 19573-19576 (1989).

Jansen, T., Rother, C., Steppuhn, J., Reinke, H., Bayreuther, K.,

Nucleotide sequence of cDNA clones encoding the complete 23kDa and 16kDa precursor proteins associated with the photosynthetic oxygen evolving complex from spinach. *Febs. Lett.* 216, 234-240 (1987).

Joyard, J., Douce, R., Import receptor in chloroplast envelope. *Nature* 333, 306-307 (1988).

Kaderbdai, M.A., Pickering, T., Austen, B.M. & Kaderbhai, N., A photoactivable synthetic transit peptide labels 30kDa and 52kDa polypeptides of the chloroplast inner envelope membrane. *Febs. Letts.* 232, 313-316 (1988).

Kaput, J., Goltz, S. & Blobel, G., Nucleotide sequence of the yeast nuclear gene for cytochrome C peroxidase precursor. *J. Biol. Chem.* 257, 15054-15058 (1982).

Kavanagh, T.A., Jefferson, R.A. & Bevan, M.W., Targeting a foreign protein to chloroplasts using fusions to the transit peptide of a chlorophyll a/b protein. *Mol. Gen. Genet.* 215, 38-45 (1988).

Keegstra, K. & Olsen, L.J., Chloroplastic precursors and their transport across the envelopes membranes. *Annu. Rev. Plant Mol. Biol.* 40, 471-501 (1989).

Kirwin, P.M., Elderfield, P.D. & Robinson, C., Transport of proteins into chloroplasts. *J. Biol. Chem.* 262, 16386-16390 (1987).

Kirwin, P.M., Elderfield, P.D., Williams, R.S., & Robinson, C., Transport of proteins into chloroplasts. *J. Biol. Chem.* 263, 18128-

18132 (1988).

Kirwin, P.M., Meadows, J.W., Shackleton, J.B., Musgrove, J.E., Elderfield, P.D., Mould, R., Hay, N.A. & Robinson, C., ATP-dependent import of a luminal protein by isolated thylakoid vesicles. EMBO J. 8, 2251-2255 (1989).

Klosgen, R.B., Saedler, H. & Weil, J.H., The amyloplast-targeting transit peptide of maize also mediates protein transport in vitro into chloroplasts. Mol. Gen. Genet. 217, 155-161 (1989).

Ko, K. & Cashmore, A.R., Targeting of proteins to the thylakoid lumen by the bipartite peptide of the 33kDa oxygen-evolving protein. EMBO J. 8, 3187-3194 (1989).

Kreig, P.A. & Melton, D.A., In vitro RNA synthesis with SP6 RNA polymerase. Methods in Enzymology 155, 397-415 (1986).

Kuwabara, T., Reddy, K.J. & Sherman, L.A., Nucleotide sequence of the gene from the cyanobacterium Anacystis nidulans R2 encoding the Mn-stabilizing protein involved in photosystem II water oxidation. Proc. Nat. Acad. Sci. USA 84, 8230-8234 (1987).

Lacoste-Royal, G. & Gibbs, S.P., Ochromonas mitochondria contain a specific chloroplast protein. Proc. Nat. Acad. Sci. USA 82, 1456-1459 (1985).

Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685 (1970).

Lamppa, G.K., The chlorophyll a/b-binding protein inserts into the thylakoids independent of its cognate transit peptide. *J. Biol. Chem.* 263, 14996-14999 (1988).

Lazdunski, C.J. & Benedetti, H., (1990). Insertion and translocation of proteins into and through membranes. *Febs. Letts.* 268, 408-414 (1990).

Lehmbeck, J., Rasmussen, O.F., Bookjans, G.B., Jepsen, B.R., Stummann, B.M. & Henningsen, K.W. Sequence of two genes in pea chloroplast DNA coding for 84 and 82 kD polypeptides of the photosystem I complex. *Plant Mol. Biol.* 7, 3-10 (1986).

Lubben, T.H., Gatenby, A.A., Ahlquist, P. & Keegstra, K., Chloroplast import characteristics of chimeric proteins. *Plant Mol. Biol.* 12, 13-18 (1989).

Mattoo, T.A. & Edelman, M., Intramembrane translocation and posttranslational palmitoylation of the chloroplast 32kDa herbicide-binding protein. *Proc. Nat. Acad. Sci. USA* 84, 1497-1501 (1987).

Meadows, J.W., Shackleton, J.B., Hulford, A. & Robinson, C., Targeting of a foreign protein into the thylakoid lumen of pea chloroplasts. *Febs. Letts.* 253, 244-246 (1989).

Messing, J., New M13 vectors for cloning. *Methods in Enzymology* 101, 20-78 (1983).

Mould, R.M. & Robinson, C. A proton gradient is required for the

transport of two lumenal oxygen-evolving proteins across the thylakoid membrane. J. Biol. Chem. 266, 12189-12193 (1991).

Olsen, L.J., Theg, S.M., Selman, B.R. & Keegstra, K., ATP is required for the binding of precursor proteins to chloroplasts. J. Biol. Chem. 264, 6724-6729 (1989).

Ou, J.W., Ito, A., Okazaki, H. & Omura, T., Purification and characterization of a processing protease from rat liver mitochondria. EMBO J. 8, 2605-2612 (1989).

Pain, D. & Blobel, G., Protein import into chloroplasts requires a chloroplast ATPase. Proc. Nat. Acad. Sci. USA, 84, 3288-3292 (1987).

Pain, D., Kanwar, Y.S. & Blobel, G., Identification of a receptor for protein import into chloroplasts and its localization to envelope contact zones. Nature 331, 232-237 (1988).

Pfaller, R., Steger, H.F., Rassow, J., Pfanner, N. & Neupert, W. Import pathways of precursor proteins into mitochondria: multiple receptor sites are followed by a common membrane insertion site. J. Cell Biol. 107, 2483-2490 (1988).

Pfanner, N. & Neupert, W., Transport of proteins into mitochondria: a potassium diffusion potential is able to drive the import of ADP/ATP carrier. EMBO J. 4, 2819-2825 (1985).

Pfanner, N., Tropschug, M. & Neupert, W., Mitochondrial protein import: nucleoside triphosphates are involved in conferring import-

competence to precursors. Cell 49, 815-823 (1987).

Pfanner, N., Hartl, F.R. & Neupert, W., Import of proteins into mitochondria: a multi-step process. Eur. J. Biochem. 175, 205-212 (1988).

Pfanner, N., Pfaller, R., Kleene, R., Ito, M., Tropscgug, M. & Neupert, W., Role of ATP in mitochondrial protein import. J. Biol. Chem. 263, 4049-4051 (1988).

Pfanner, N., Sollner, T. & Neupert, W. Mitochondrial import receptors for precursor proteins. Trends in Biol. Sci.. 16, 63-67 (1991).

Pfisterer, J., Lachmann, P. & Kloppstech, K., Transport of proteins into chloroplasts. Eur. J. Biochem. 126, 143-148 (1982).

Pilon, M., de Boer, A.D., Knols, S.L., Koppelman, M.H.G.M., van der Graaf, R.M., Kruijff, B. & Weisbeck, P.J., Expression in E.coli and purification of a translocation-competent precursor of the chloroplast protein ferredoxin. J. Biol. Chem. 265, 3358-3361 (1990).

Pugsley, A.P., Protein targeting. Academic press, Inc. P. 1-12 (1989).

Puoti, A., Desponds, C. & Conzelmann, A. Biosynthesis of mannosylinositolphosphoceramide in Saccharomyces cerevisiae is dependent on genes controlling the flow of secretory vesicles from the endoplasmic reticulum to the Golgi. J. Cell Biology 113, 515-525 (1991).

Rapoport, T.A., Protein transport across the ER membrane. Trends in Biol. Sci. 15, 355-358 (1990).

Reinbothe, S., Krauspe, R. & Parthier, B., In vitro transport of chloroplast proteins in a homologous Euglena system with particular reference to plastid leucyl-tRNA synthetase. Planta 181, 176-183 (1990).

Reiss, B., Wasmann, C.C., Schell, J. & Bohnert, H.J., Effect of mutations on the binding and translocation function of a chloroplast transit peptide. Proc. Nat. Acad. Sci. USA 86, 886-890 (1989).

Roberts, B.E. & Patterson, B.M., Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. Proc. Nat. Acad. Sci. USA 70, 2330-2334 (1974).

Robinson, C. & Ellis, R.J., Transport of proteins into chloroplasts. Eur. J. Biochem. 142, 337-342 (1984).

Roise, D., Horvath, S.J., Tomich, J.M., Richards, J.H. & Schatz, G., A chemically synthesised pre-sequence of an imported mitochondrial protein can form an amphiphilic helix and perturb natural and artificial phospholipid bilayers. EMBO J. 5, 1327-1334 (1986).

Roise, D., Theiler, F., Horvath, S.J., Tomich, J.M., Richards, J.H., Allison, D.S. & Schatz, G., Amphiphilicity is essential for mitochondrial pre-sequence function. EMBO J. 7, 649-653 (1988).

Romisch, J., Tropschug, M., Sebald, W. & Weiss, H., The primary

structure of cytochrome c_1 from Neurospora crassa. Eur. J. Biochem. 164, 111-115 (1987).

Rothblatt, J.A. & Meyer, D.I., Secretion in yeast: translocation and glycosylation of prepro-alpha-factor in vitro can occur via an ATP-dependent post-translational mechanism. EMBO J. 5, 1031-1036 (1986).

Sadler, I., Suda, K. Schatz, G., Kausewitz, F. & Haid, A. Sequencing of the nuclear gene for the yeast cytochrome C_1 precursor reveals an unusually complex amino-terminal pre-sequence. EMBO. J. 3, 2137-2143 (1984).

Salomon, C., Fisker, K., Flugge, U.I. & Soll, J., Sequence analysis and import studies of an outer envelope polypeptide. Proc. Nat. Acad. Sci. USA 87, 5778-5782 (1990).

Sambrook, J., Fritsch, E.F. & Maniatis, T., Molecular cloning A laboratory manual Second edition. Cold Spring Harbor Laboratory press (1989).

Sanger, F., Nicklen, S. & Coulson, A.R., DNA sequencing with chain terminating inhibitors. Proc. Nat. Acad. Sci. USA 74, 5463-5467 (1977).

Saraste, J. & Kuismanen, E. Pre- and post-golgi vacuoles operate in the transport of semliki forest virus membrane glycoproteins to the cell surface. Cell 38, 535-549 (1984).

Schneider, A., Behrens, M., Scherer, P., Pratje, E., Michaelis, G. &

Schatz, G., Inner membrane protease I, an enzyme mediating intramitochondrial protein sorting in yeast. EMBO J. 10, 247-254 (1991).

Schnell, D.J., Blobel, G. & Pain, D. the chloroplast import receptor is an integral membrane protein of chloroplast envelope contact sites. J. Cell Biol. 111, 1825-1838 (1990).

Schnepf, E., Chloroplasts. Ed. J.Reinart. Springer J., 4, 25-32 (1980).

Schwartz, R.M. & Dayhoff, M.O., Origins of prokaryotes, eukaryotes, mitochondria and chloroplasts. Science 199, 395-403 (1978).

Seidler, A. & Michel, H., Expression in E.coli of the psbO gene encoding the 33kd protein of the oxygen-evolving complex from spinach. EMBO J. 9, 1743-1748 (1990).

Smeekeens, S., de Groot, M., van Binsbergen, J. & Weisbeek, P., Sequence of the precursor of the chloroplast thylakoid lumen protein plastocyanin. Nature 317, 456-458 (1985).

Smeekeens, S., Bauerle, J.H., Hageman, J., Keegstra, K. & Weisbeek, P., The role of the transit peptide in the routing of precursors toward different chloroplast compartments. Cell 46, 365-375 (1986).

Smeekeens, S., van Steeg, H., Bauerle, C., Bettenbroek, H., Keegstra, K. & Weisbeek, P., Import into chloroplasts of a yeast mitochondrial protein directed by ferredoxin and plastocyanin transit peptides. Plant

Mol. Biol. 9, 377-388 (1987).

Smeekens, S. & Weisbeek, P., Protein transport towards the thylakoid lumen: post-translational translocation in tandem. Photosyn. Res. 16, 177-186 (1988).

Soll, J. & Buchanan, B.B., Phosphorylation of chloroplast ribulose biphosphate carboxylase/oxygenase small subunit by an envelope-bound protein kinase in situ. J. Biol. Chem. 258, 6686-6689 (1983).

Soll, J., Purification and characterization of a chloroplast outer-envelope-bound, ATP-dependent protein kinase. Plant Physiol. 87, 898-903 (1988).

Soll, J., Fischer, I. & Keegstra, K., A guanosine 5'-triphosphate-dependent protein kinase is localized in the outer envelope membrane of pea chloroplasts. Planta 176, 488-496 (1988).

Tamm, L.K., Incorporation of a synthetic mitochondrial signal peptide into charged and uncharged phospholipid monolayers. Biochemistry 25, 7470-7476 (1986).

Theg, S.M., Bauerle, C., Olsen, L.J., Selman, B.R. & Keegstra, K., Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes. J. Biol. Chem. 264, 6730-6736 (1989).

Tokunaga, M., Loranger, J.M., Wolfe, P.B. & Wu, H.C., Polypeptide signal peptidase in Escherichia coli is distinct from the M13 procoat

protein signal peptidase. *J. Biol. Chem.* 257, 9922-9925 (1982).

Tyagi, A., Hermans, J., Steppuhn, J., Jansson, C., Vater, F. & Herrmann, R.G., Nucleotide sequence of cDNA clones encoding the complete "33kDa" precursor protein associated with the photosynthetic oxygen-evolving complex from spinach. *Mol. Gen. Genet.* 207, 288-293 (1987).

Van den Broeck, G., Timko, M.P., Kausch, A.P., Cashmore, A.R., Van Montagu, M., & Herrera-Estrella, L., Targeting of a foreign protein to chloroplasts by fusion to the transit peptide from the small subunit of ribulose 1,5-bisphosphate carboxylase. *Nature* 313, 353-363 (1985).

Verner, K. & Schatz, G., Protein translocation across membranes. *Science* 241, 1307-1313 (1988).

Viitanen, P.V., Doran, E.R. & Dunsmuir, P., What is the role of the transit peptide in thylakoid intergration of the light-harvesting chlorophyll a/b protein. *J. Biol. Chem.* 263, 15000-15007 (1988).

Von Heijne, G., Signal sequences the limits of variation. *J. Mol. Biol.* 184, 99-105 (1985).

Von Heijne, G., Steppuhn, J. & Herrmann, G., Domain structure of mitochondrial and chloroplast targeting peptides. *Eur. J. Biochem.* 180, 535-545 (1989).

Walter, P. & Lingappa, V.R. Mechanism of protein translocation across the endoplasmic reticulum membrane. *Ann. Rev. Cell Biol.* 2, 499-516

(1986).

Waters, M.G. & Blobel, G., Secretory protein translocation in a yeast cell-free system can occur posttranslationally and requires ATP hydrolysis. *J. Cell Biol.* 102, 1543-1550 (1986).

Webber, A.N., Packman, L.C. & Gray, J.C., A 10kDa polypeptide associated with the oxygen-evolving complex of photosystem II has a putative C-terminal non-cleavable thylakoid transfer domain. *Febs. Letts.* 242, 435-438 (1989).

Whelan, J., Knorpp, C. & Glaser, E., Sorting of precursor proteins between isolated leaf mitochondria and chloroplasts. *Plant Mol. Biol.* 14, 977-982 (1990).

Whitmarsh, J., Photosynthesis (Encyclopaedia of plant physiol). Ed. Staehelin, L.A. & Arntzan, C.J. Springer Verlag, 508-527 (1986).

Wickner, W., The assembly of proteins into biological membranes: the membrane trigger hypothesis. *Annu. Rev. Biochem.* 48, 23-45 (1979).

Wickner, W.T. & Lodish, H.F. Multiple mechanisms of protein insertion into and across membranes. *Science* 230, 400-407 (1985).

Witte, C., Jensen, R.E., Yaffe, M.P. & Schatz, G., MAS1, a gene essential for yeast mitochondrial assembly, encodes a subunit of the mitochondrial processing protease. *EMBO J.* 7, 1439-1447 (1988).

Wolfe, P.B., Wickner, W. & Goodman, J.M., Sequence of the leader

peptidase gene of E.coli and the orientation of leader peptidase in the bacterial envelope. J. Biol. Chem. 258, 12073-12080 (1983).

Yamamoto,N., Alberts,B.M., Benzinger,R., Lawthorne, L. & Treiber, G., Rapid bacteriophage sedimentation in the presence of Polyethylene Glycol and its application to large scale virus purification. Virology 40, 734-744 (1970).

Young, R.A. & Davies, R.W. Efficient isolation of genes by using antibody probes. Proc. Nat. Acad. Sci. USA 80, 1194-1198 (1983).

Zimmermann,R, Watts,C. & Wickner,W. The biosynthesis of membrane-bound M13 coat protein. J. Biol. Chem. 257 ,6529-6536 (1982).

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